Upregulation of Ca\(^{2+}\) removal in human skeletal muscle: a possible role for Ca\(^{2+}\)-dependent priming of mitochondrial ATP synthesis

Werner J. H. Koopman,1,2 Michel Renders,2 Arie Oosterhof,2 Toin H. van Kuppevelt,2 Baziel G. M. van Engelen,3 and Peter H. G. M. Willems1,2

1Microscopical Imaging Center and 2Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen; and 3Institute of Neurology, Neuromuscular Center Nijmegen, University Medical Center Nijmegen, NL-6500 HB Nijmegen, The Netherlands

Submitted 10 March 2003; accepted in final form 26 June 2003

Koopman, Werner J. H., Michel Renders, Arie Oosterhof, Toin H. van Kuppevelt, Baziel G. M. van Engelen, and Peter H. G. M. Willems. Upregulation of Ca\(^{2+}\) removal in human skeletal muscle: a possible role for Ca\(^{2+}\)-dependent priming of mitochondrial ATP synthesis. Am J Physiol Cell Physiol 285: C1263–C1269, 2003. First published July 2, 2003; 10.1152/ajpcell.00097.2003.—In muscle, ATP is required for the powerstroke of the myosin head, the detachment of actin and myosin filaments, and the reuptake of Ca\(^{2+}\) into the sarcoplasmic reticulum. During contraction-relaxation, large amounts of ATP are consumed at the sites of action of the myosin-ATPase and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. The present study addresses the consequences of a reduction in mitochondrial ATP production capacity on sarcoplasmic Ca\(^{2+}\) handling. To this end, myotubes were cultured from patient quadriceps with a biochemically defined decrease in the maximal rate of glycolysis. Myotubes were field-stimulated with 10-ms pulses of tricuspid stimulation; mitochondrial memory for sarcoplasmic [Ca\(^{2+}\)] was long-lasting and independent of the strength of the stimulus. In myotubes with a reduced mitochondrial ATP production capacity, the amplitude of the [Ca\(^{2+}\)] transients was markedly decreased compared with control. Moreover, upregulation of the CRR was significantly decreased compared with control. Taken together, these results are in good agreement with a tight coupling between mitochondrial ATP production and sarcoplasmic Ca\(^{2+}\) handling. Moreover, they support the existence of a relatively long-lasting mitochondrial memory for sarcoplasmic Ca\(^{2+}\) removal. This memory, which manifested itself as an increase in CRR upon recurrent stimulation, was impaired in patient myotubes with a reduced mitochondrial ATP production capacity.

Changes in sarcoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{S}}\)) are the primary driving force for muscle contraction/relaxation and regulate gene expression and muscle differentiation (4, 26, 29, 30, 42). In skeletal muscle, excitation-contraction (EC) coupling is achieved by a functional and physical interaction between the dihydropyridine receptors (DHPR) in the transverse tubules and Ca\(^{2+}\) release channels (ryanodine receptors type 1 or RyR1) in the junctional terminal cisternae of the sarcoplasmic reticulum (SR; Ref. 10). The resulting increase in [Ca\(^{2+}\)]\(_{\text{S}}\) is dissipated by the action of sarcoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs), which resequester the released Ca\(^{2+}\) into the SR. Abnormalities in the EC system, like mutations in RyR or adult fast-twitch muscle SR Ca\(^{2+}\)-ATPase isoform (SERCA1a), lead to muscle dysfunction (24). In contrast to cardiac muscle, EC coupling in skeletal fibers does not require influx of extracellular Ca\(^{2+}\) nor Ca\(^{2+}\)-induced Ca\(^{2+}\) release (3, 4, 7). During the transition from rest to high-intensity exercise, the ATP consumption of skeletal muscle cells can increase up to 100-fold (41). This ATP is not only spent on myofilament sliding but it is also required to drive SERCA pumping. Experimental data demonstrate that the latter pump can utilize up to 55% of total ATP production in human skeletal muscle fibers (37). Finally, activation of the ryanodine receptor (RyR) by the DHPR has been shown to require ATP binding (20).

The central role of [Ca\(^{2+}\)]\(_{\text{S}}\) dynamics in EC coupling, and its dependence on ATP, requires a tight balance between ATP production and demand. In skeletal muscle, this balance is achieved by a direct Ca\(^{2+}\)-mediated stimulation of mitochondrial ATP production (17). The mechanism involves mitochondrial Ca\(^{2+}\) uptake stimulating the activity Ca\(^{2+}\)-sensitive dehydrogenases, including the pyruvate dehydrogenase complex (PDHC), 2-oxoglutarate dehydrogenase, and NAD\(^{+}\)-isocitrate dehydrogenase and possibly also elements of the electron transport chain, the ATP synthase itself, and the adenine nucleotide translocase (11, 28, 32, 33). As a consequence, the production of ATP will increase (1, 5, 17), allowing a higher energy flux through the creatine kinase (CK) and adenylate kinase (AK) relay systems (9, 40, 43). Disturbances in these systems, studied in

Address for reprint requests and other correspondence: P. H. G. M. Willems, 160 Biochemistry NCMLS, Univ. Medical Center Nijmegen, PO Box 9101, NL-6500 HB Nijmegen, The Netherlands (E-mail: p.willems@ncmls.kun.nl).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpcell.org 0363-6143/03 $5.00 Copyright © 2003 the American Physiological Society C1263
CK and AK knockout animals, result in impaired tectonic force output, increased relaxation times, and aberrant sarcoplasmic Ca^{2+} dynamics (15, 35). Saks and coworkers (34) suggested the existence of a functional complex consisting of mitochondria, SERCAs, and SR. Obviously, such a complex would facilitate optimal mitochondrial ATP production and CK/AK-mediated channeling of ATP to the SERCA pumps. Using CK-knockout mice, we recently demonstrated that a functional CK system is required for optimal refill of the SR (7). This study aims to gain insight into the interdependence of sarcoplasmic Ca^{2+} signaling and mitochondrial ATP production in human skeletal muscle. For this purpose, we analyzed sarcoplasmic Ca^{2+} concentration ([Ca^{2+}]_{s}) dynamics in myotubes derived from human quadriceps muscle biopsies with a defective mitochondrial ATP production. Myotubes were loaded with indo 1, and electrically induced changes in sarcoplasmic Ca^{2+} concentration were monitored by means of video-rate confocal microscopy. It was observed that, upon recurrent stimulation, the rate of sarcoplasmic Ca^{2+} removal became significantly faster in control myotubes and that this upregulation was impaired in myotubes having mitochondria with a reduced ATP production capacity.

**MATERIALS AND METHODS**

**Muscle biochemistry.** Quadriceps needle muscle biopsies (25–50 mg) were obtained from a healthy volunteer (control myotubes) and two patients with an adult onset of exercise intolerance and exercise-induced myalgia and stiffness (myotubes A and B), as approved by the Committee on Medical Ethics. After homogenization of fresh muscle tissue, a 600-g supernatant was prepared for biochemical analysis of disturbances in the mitochondrial energy generating system (procedure described in Ref. 14). Briefly, the overall activity of the energy supplying system was determined by measuring the oxidation rates of [14C]-labeled pyruvate, malate, and succinate and the ATP-production rate from the oxidation of pyruvate. In addition, the activities of single enzymes from the respiratory chain and citrate synthase were measured. Table 1 demonstrates that in both biopsies the production rate of ATP and the oxidation rates of malate and succinate were reduced, whereas the activities of citrate synthase and the respiratory chain enzymes were normal.

**Culture of myotubes.** Muscle samples from the patients and a healthy subject were dissociated according to the method of Yasin et al. (44) as described previously (39). Briefly, muscle samples were incubated four times for 15 min in 20 ml of Dulbecco’s phosphate-buffered saline containing 30 mg trypsin, 15 mg collagenase, and 15 mg albumin. Satellite cells were spun down at 50 g, resuspended in Dulbecco’s modified Eagles medium (DMEM; Invitrogen, Breda, The Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), and allowed to proliferate for 4–5 days. Aliquots of the primary culture were stored in liquid nitrogen. For each measurement, one patient and one control aliquot was plated on four glass coverslips each and cultured to confluence in DMEM with 20% FCS. One day after reaching confluence, the medium was replaced by DMEM supplemented with 0.4% Ultrorser G (Invitrogen) to promote myoblast fusion into myotubes.

Table 1. Biochemistry of quadriceps muscle

<table>
<thead>
<tr>
<th>Biochemical Parameter</th>
<th>Myotubes A</th>
<th>Myotubes B</th>
<th>Control Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidation of</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[14C]pyruvate + carnitine</td>
<td>3.81</td>
<td>5.49</td>
<td>2.84–8.24</td>
</tr>
<tr>
<td>[14C]malate + pyruvate + malonate</td>
<td>3.54</td>
<td>4.57</td>
<td>4.68–9.62</td>
</tr>
<tr>
<td>[14C]malate + acetyl carnitine + malonate</td>
<td>2.42</td>
<td>3.38</td>
<td>3.43–7.30</td>
</tr>
<tr>
<td>[14C]malate + acetyl carnitine + arsenite</td>
<td>1.35</td>
<td>1.64</td>
<td>2.05–3.85</td>
</tr>
<tr>
<td>[14C]succinate + acetylcarnitine</td>
<td>1.62</td>
<td>2.08</td>
<td>2.54–6.39</td>
</tr>
<tr>
<td><strong>ATP production from</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate oxidation</td>
<td>26†</td>
<td>32</td>
<td>42–81</td>
</tr>
<tr>
<td>Single enzyme activities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH-Q1 oxidoreductase (CI)</td>
<td>113‡</td>
<td>87</td>
<td>70–250</td>
</tr>
<tr>
<td>Succinate dehydrogenase (CII)</td>
<td>104</td>
<td>139</td>
<td>67–177</td>
</tr>
<tr>
<td>Decyubiquinol:cyt c oxidoreductase (CIII)</td>
<td>2,960</td>
<td>2,733</td>
<td>2,200–6,610</td>
</tr>
<tr>
<td>Cytochrome c oxidase (CIV)</td>
<td>1,027</td>
<td>1,728</td>
<td>883–3,120</td>
</tr>
<tr>
<td><strong>Citrate synthase</strong></td>
<td>130§</td>
<td>107</td>
<td>37–162</td>
</tr>
</tbody>
</table>

*Substrate oxidation values are expressed as nmol CO₂/mU citrate synthase/hour; †ATP production values are expressed as nmol ATP/mU citrate synthase/hour; ‡single enzyme activities are expressed as mU per unit of citrate synthase; §citrate synthase activity is expressed as mU per milligram of protein. Muscle biochemistry was performed in a 600-g supernatant of fresh quadriceps muscle homogenate according to assays described in Jansen et al. (14).
confocal detection volume (section thickness of 5–8 μm; Ref. 18) and enabled us to run the laser at minimal levels (output of 15 mW, equivalent to 28 μW at the back of the objective lens). Basal ratio signals were stable, and no UV-induced deterioration in cell viability was observed during the experiments. The OZ hardware was controlled by the Intervision acquisition software (version 1.5, Noran Instruments) running under IRIX 6.5 on an Indy workstation (Silicon Graphics, Mountain View, CA) equipped with 256 Mb of internal memory. Before the onset of the measurement, six regions of interest distributed throughout the sarcoplasm of the myotube were indicated. For background correction, an additional region of interest was situated outside the myotube. During the measurement, the average fluorescence emission intensities from each region of interest were calculated and individually stored in ASCII format for offline background correction and ratioing.

**Electrical stimulation.** For electrical stimulation, two platinum electrodes were fixed to opposite sides of the chamber and connected to a Grass SD9 stimulator. Myotubes were stimulated (10 ms, 16 V) at the frequencies indicated. All recordings were performed at room temperature on at least three different days. Of note, myotubes were not spontaneously active, and electrically induced [Ca²⁺]₅₆ rises were observed only in multinucleated myotubes and not in mononucleated myoblasts.

**Data analysis.** The ASCII files were imported in Origin Pro 6.1 (Microcal) for offline analysis. Emission signals were background-corrected, and the fluorescence emission ratio (405/485 nm) was calculated. For quantitative analysis, ratio signals were normalized relative to the prestimulatory indo 1 transient increase in [Ca²⁺]₅₆. Between successive transients, [Ca²⁺]₅₆ was allowed to return to prestimulatory levels (dotted line). For a single stimulus, statistical analysis was carried out using SPSS 11 (SPSS, Chicago, IL). To assess whether experimental data displayed a normal distribution, the Kolmogorov-Smirnov test with Lilliefors significance correction was applied. Linear fits were tested for significance using a Student’s t-test (P < 0.01).

**RESULTS**

**Electrically induced Ca²⁺ transients in human myotubes.** In this study, we compared sarcoplasmic Ca²⁺ kinetics ([Ca²⁺]₅₆) between myotubes cultured from quadriceps muscle with a normal (“control,” n = 43) and reduced (“A myotubes,” n = 42 and “B myotubes,” n = 58) mitochondrial ATP production (Table 1). Myotubes were loaded with the ratiometric Ca²⁺-indicator indo 1, and changes in [Ca²⁺]₅₆, induced by electrical field stimulation, were monitored in randomly chosen regions of interest (ROIs) in the sarcoplasm. The kinetics of the [Ca²⁺]₅₆ appeared to be independent of the exact localization of this region (data not shown). To impose different Ca²⁺ loads, a protocol was applied that consisted of three single stimuli (1, 2, and 3) followed by two trains of 10 stimuli at 1 Hz (4 and 5) and a final single stimulus (6; Fig. 1A, data from a representative control myotube). The stimuli induced transient increases in [Ca²⁺]₅₆.

---

**Fig. 1.** Kinetic analysis of electrically induced sarcoplasmic Ca²⁺ concentration ([Ca²⁺]₅₆) transients in cultured human myotubes. A: electrical stimulation protocol (lower trace) and corresponding changes in [Ca²⁺]₅₆ (upper trace) in a control myotube. Five stimuli were applied in succession: 3 of low intensity (single pulses 1, 2, and 3), 2 of high intensity (pulse trains 4 and 5), and a final 1 of low intensity (single pulse 6). The frequency of the pulse trains was 1 Hz. B: kinetic parameters used for quantitative analysis of electrically induced [Ca²⁺]₅₆ transients. For each stimulus, its amplitude A [given by Rmax/prestimulatory indo 1 ratio (R₀)] and time constant μ (determined from an exponential fit; see RESULTS) were determined. The trace shown is a representative example of myotubes B. C: quantification of changes in amplitude A during electrical stimulations. In the typical example shown (where A₁ is plotted as a function of A₀ for myotubes B), each data point corresponds to a different myotube and the linear fit (dotted line) lies above y = x (thick line). The parameter ΔA (difference in amplitude) is used to quantify the latter difference (for details, see MATERIALS AND METHODS). D: similar to C, μ₁ was plotted as a function of μ₀ for myotubes B. For convenience, Δμ was defined as 1 minus the slope of the linear fit so that an increase in Δμ reflects an increase in Ca²⁺ removal rate.
[Ca\(^{2+}\)]_s increased from the baseline ratio to a maximum within 33 ms. This increase was followed by a more gradual decline to prestimulatory levels. During pulse trains, [Ca\(^{2+}\)]_s increased to a plateau level. After the last pulse, [Ca\(^{2+}\)]_s gradually declined to baseline.

**Kinetic analysis of Ca\(^{2+}\) transients.** After normalization, the kinetics of the decay was determined and appeared to be adequately described by a monoexponential function: \(R(t) = R_{\text{max}} e^{-t/\mu} + A\), where \(R(t)\) is the indo 1 ratio at time \(t\), \(R_{\text{max}}\) is the indo 1 ratio at the onset of the [Ca\(^{2+}\)]_s decline, \(\mu\) is the time constant, which is inversely proportional to the Ca\(^{2+}\) removal rate (CRR), and \(A\) is the value to which the indo 1 ratio declines. The latter parameter proved to be the prestimulatory indo 1 ratio (\(R_0\)). During curve fitting, the robust Levenberg-Marquardt algorithm was used and \(R^2\), representing the coefficient of determination, was used to express the proportion of total variation that is explained by the regression. If \(R^2\) is \(\leq 1\), the total variation in the \(R(t)\) can be explained in terms of the regression curve (7). In our analysis, only fits with \(R^2 > 0.9\) were included. The monoexponential kinetics of the [Ca\(^{2+}\)]_s decay demonstrates the action of a single Ca\(^{2+}\) removal mechanism (7, 16, 18, 19, 22, 38). The time constant \(\mu\) was used as a quantitative readout of CRR (Fig. 1B, data from a representative myotube B). For each myotube, six time constants (\(\mu_1\) to \(\mu_6\)) were calculated: \(\mu_1\) to \(\mu_3\) for the first three single pulses, \(\mu_4\) and \(\mu_5\) for the subsequent two 1-Hz pulse trains, and \(\mu_6\) for the last single pulse. Similarly, the maximum amplitudes \(A_1\) to \(A_6\) (given by \(R_{\text{max}}\) of the corresponding [Ca\(^{2+}\)]_s transient) were determined.

Unfortunately, the distribution of amplitude and time constants appeared to be non-Gaussian (\(P < 0.05\)) for the majority of the pulses. This precluded use of common statistical significance tests. In addition, the time constant \(\mu\) for a given stimulus appeared to be highly variable between individual myotubes, even when present on the same coverslip. For example, \(\mu_1\) varied from 2.01 to 20.4 s (control), 1.88 to 15.4 s (myotubes A), and 1.75 to 10.7 s (myotubes B). To resolve these issues, \(A_2\), \(A_3\), \(A_4\), \(A_5\), and \(A_6\) were plotted as a function of \(A_1\). The rationale behind this method is illustrated in Fig. 1C, where \(A_4\) is plotted as a function of \(A_1\) for all myotubes B. First, a straight line was fitted (dotted line) and its slope was determined. Next, it was tested whether the confidence interval of this slope did or did not include 1. If the latter was the case, and if the linear model was appropriate (\(P < 0.01\); observed for all scatter plots), the fitted line was considered to be significantly different from the line \(y = x\) (thick line). Calculation of the upper and lower confidence limits of the slope allows statistical comparison between different stimuli and populations of myotubes (23). A slope larger (smaller) than 1 indicates a higher (lower) amplitude relative to the first stimulus. By subtracting 1 from the slope of the fitted line, a \(\Delta\mu\) value was obtained, which when positive (negative) represents an increase (decrease) in amplitude. Similarly, intermyotube variation in \(\mu\) was corrected. For reasons of clarity, the value of the slope of the fitted line was subtracted from 1, yielding a \(\Delta\mu\) that changes in the same direction as CRR (Fig. 1D, data from myotubes B). This approach considers the collective change in Ca\(^{2+}\) handling in a population of myotubes and condenses these changes into two parameters (\(\Delta\mu\) and \(\Delta\mu\)).

**Dependence of Ca\(^{2+}\) kinetics on stimulus intensity.** The results of the kinetic analysis are depicted in Fig. 2. In control myotubes (Fig. 2A, solid bars), \(\Delta\mu\) did not differ from zero for all stimuli. This indicates that the amplitude of the transients was always maximal and independent of stimulus intensity. For myotubes A (hatched bars) and B (open bars), \(\Delta\mu\) was zero for stimuli 2 and 3. At higher stimulus intensities (stimuli 4 and 5), however, the amplitude was significantly increased (\(A_4\) and \(A_5\); upward arrowheads indicate significant increases). For the last stimulus, \(\Delta\mu\) was

---

**Fig. 2.** Dependence of amplitude and Ca\(^{2+}\) removal rate on stimulus intensity in control myotubes and myotubes with a reduced mitochondrial ATP production capacity. A: \(\Delta\mu\) between the first and subsequent [Ca\(^{2+}\)]_s rises. \(\Delta\mu\) is calculated as described in Fig. 1. The data presented are from control (solid bars), myotubes A (hatched bars), and myotubes B (open bars). B: difference in CRR (\(\Delta\mu\)) between the first and subsequent [Ca\(^{2+}\)]_s rises. \(\Delta\mu\) is calculated as described in Fig. 1. The data presented are from control (solid bars), myotubes A (hatched bars), and myotubes B (open bars). Up- and downward arrowheads represent significant increases and decreases, respectively. The confidence interval of the slope of the linear fit (see Fig. 1) is given by error bars. In case error bars do not overlap, differences are considered significant (\(P < 0.05\)). *ct, Significantly different from corresponding control.
negative, indicating that $A_b$ was significantly smaller than $A_1$.

Figure 2B (solid bars) shows that $\Delta \mu$ was significantly higher after the second stimulus in control cells. For successive stimuli, no further change in $\Delta \mu$ was observed. These data demonstrate that CRR is upregulated during repeated stimulation and that this upregulation is already maximal at the second stimulus. In myotubes A (hatched bars) and B (open bars), the increase in $\Delta \mu$ was significantly lower than in control for the second stimulus. For myotubes A, $\Delta \mu$ no longer increased for stimulus 3, similar to control for pulse trains 4 and 5, and not increased for stimulus 6. In contrast, $\Delta \mu$ remained virtually the same for myotubes B.

**DISCUSSION**

The present study reports the consequences of a decrease in mitochondrial ATP production capacity on sarcoplasmic Ca$^{2+}$ handling in human myotubes. The major findings are that, relative to control, myotubes with a decrease in mitochondrial ATP production capacity show a less profound increase in CRR upon recurrent stimulation, and, in contrast to control, these myotubes display sarcoplasmic Ca$^{2+}$ rises, the amplitude of which increases significantly with stimulus intensity.

Electrically induced [Ca$^{2+}$]$_S$ transients in human skeletal myotubes. Myotubes were cultured from quadriceps needle muscle biopsies, obtained from a healthy volunteer (control myotubes) and two patients with an adult onset of exercise intolerance and exercise-induced myalgia and stiffness (myotubes A and B). Biochemical analysis of fresh muscle homogenates revealed maximal ATP production rates that were 40% (myotubes A) and 25% (myotubes B) below the lowest control value. Transient increases in [Ca$^{2+}$]$_S$, closely mimicking the physiological situation, were evoked by short periods (10 ms) of electrical field stimulation (7, 16). Changing the intensity of the electrical field stimulation allowed us to study the dynamics of [Ca$^{2+}$]$_S$ at different sarcoplasmic Ca$^{2+}$ loads. A single stimulus caused an increase in [Ca$^{2+}$]$_S$, which reached its maximum amplitude within 33 ms and declined thereafter to prestimulatory levels within 15 s. Earlier studies have shown that the changes in [Ca$^{2+}$]$_S$ measured in these myotubes originate from SR-mediated Ca$^{2+}$ release (3). Together with the observation that electrical stimulation did not induce [Ca$^{2+}$]$_S$ changes in myoblasts, this indicates that DHPRs and RyRs are functionally coupled in these myotubes. All transients displayed monoexponential decay kinetics, indicating the involvement of a single removal mechanism (18, 19, 22, 38). Using mouse skeletal myotubes, we have previously implicated the SERCA pump in this mechanism (7).

Stimulus-induced upregulation of Ca$^{2+}$ removal in control myotubes. In control cells, the CRR increased by ~50% when going from the first to the second single stimulus. Because sarcoplasmic Ca$^{2+}$ removal occurs primarily through the SERCA (see above), this implies a marked increase in SERCA pumping. The activity of this pump depends, among others, on the cytosolic Ca$^{2+}$ concentration. However, the amplitude of the [Ca$^{2+}$]$_S$ transient was not different between the two consecutive stimuli. Therefore, CRR upregulation must be mediated by another mechanism. Here, we postulate that CRR upregulation is the result of increased supply of phosphocreatine and/or ATP to the SERCA pumps. According to this hypothesis, resting myotubes have a reduced availability of phosphocreatine and/or ATP at the sites of SERCA pumping, thus explaining the relatively low CRR of the first single stimulus.

It is well established that Ca$^{2+}$ ions rapidly enter the mitochondrial matrix when the cytosolic Ca$^{2+}$ concentration increases (12, 28, 31, 36). Rizzuto and colleagues (17) have shown that such an increase in mitochondrial Ca$^{2+}$ concentration leads to long-lasting priming of mitochondrial metabolism in skeletal myotubes. It was proposed that this mitochondrial memory, which lasted longer than the sarcoplasmic and mitochondrial [Ca$^{2+}$]$_S$ transients, allows mitochondria to meet the increased energy requirements of a stimulated cell with no risk of futile Ca$^{2+}$ cycling and/or organelle Ca$^{2+}$ overload. Based on these literature findings, we postulate that the mitochondrial Ca$^{2+}$ increase that occurs during the first stimulus primes the mitochondrial ATP production machinery for subsequent stimulations. As a result, more phosphocreatine and/or ATP will be available for the SERCA pumps during the second and next stimuli. This idea is in agreement with earlier studies in skeletal muscle, showing that the mitochondrial uncoupler FCCP induces a decrease in CRR (21).

Despite a marked increase in CRR, the amplitude of the [Ca$^{2+}$]$_S$ rise did not increase when going from the first to the second single stimulus in control myotubes. This demonstrates that the rate at which Ca$^{2+}$ is released from the SR during the short period of depolarization (10 ms) exceeds by far the CRR. When changing from the third single stimulus to the first pulse train, the maximal amplitude tended to increase but the effect was not statistically significant. In the case of a train of pulses given at 1 Hz, the increase in [Ca$^{2+}$]$_S$ evoked by the first pulse was considerably higher than that evoked by the second pulse, whereas the increase evoked by the third and subsequent pulses was similar to that evoked by the second pulse. This suggests that either the Ca$^{2+}$ release process is inhibited during repeated stimulation (see also Refs. 8, 27) or the CRR is sufficiently high to resequester the Ca$^{2+}$ that is released during the preceding release event. The latter explanation implies that sufficient amounts of ATP are produced and delivered to the SERCA pumps. The observation that more Ca$^{2+}$ accumulates in the mitochondrial matrix during high frequency stimulation (21) is in agreement with the proposed increase in ATP production. It is thought that inorganic phosphate, released in large quantities upon ATP hydrolysis, enters the SR and causes precipitation
of calcium phosphate. This precipitation reaction is held responsible for the reduction in SR Ca\(^{2+}\) release during high-intensity muscle activity (2). On the other hand, increases in inorganic phosphate in the sarcoplasm have been reported to increase the open probability of RyRs and to slow down SERCA pumping (8). Obviously, the latter finding does not agree with the increase, rather than decrease, in CRR observed during the two pulse trains in this study.

**Aberrant Ca\(^{2+}\) handling in ATP-deficient myotubes.** Upon first stimulation, the CRR was only moderately increased (~20%) in myotubes with a reduced mitochondrial ATP production capacity. According to our hypothesis, this finding reflects a disturbed coupling between ATP consumption and ATP supply at the sites of active Ca\(^{2+}\) pumping. The observation that both the production of ATP and the oxidation of malate and succinate were decreased in a homogenate of the corresponding fresh muscle biopsies suggests that the production, and not distribution, of ATP is rate limiting in the patient-derived myotubes. A decreased supply of ATP to the sites of action not only hampers ATP-dependent sarcoplasmic Ca\(^{2+}\) removal (7) but also ATP-dependent detachment of the myosin head from the actin filament. This may very well explain the muscle stiffness observed in the subjects from whom myotubes A and B were derived (unpublished data) and agrees with earlier studies in *mdx* mouse myotubes and *Xenopus laevis* skeletal muscle fibers, showing that a decrease in Ca\(^{2+}\) removal capacity affects muscle relaxation (25, 42).

Myotubes A showed a down- rather than upregulation of the CRR when changing from the second to the third single stimulus in succession. This indicates that these myotubes lack a mitochondrial memory. In contrast to control myotubes, myotubes A showed an additional increase in \([\text{Ca}^{2+}]_s\) during the second and third pulse of the pulse train. As a result, the amplitude significantly increased when changing from the third single stimulus to the first pulse train. This suggests that the CRR was not sufficiently upregulated to balance Ca\(^{2+}\) release during the first three pulses of the pulse train. A marked increase in CRR was observed at the end of the two pulse trains. According to our hypothesis, this means a substantial increase in mitochondrial ATP production. Apparently, however, this increase was not sufficient to prevent the amplitude from increasing. When changing from the second pulse train to the last single pulse, the CRR immediately dropped. Again, this is in agreement with the absence of a mitochondrial memory.

In myotubes B, the CRR hardly changed when going from the second to the third single stimulus. This indicates that these myotubes, in contrast to myotubes A, do have a mitochondrial memory. In contrast to myotubes A, myotubes B showed no increase in CRR at the end of the first pulse train. According to our hypothesis, this means that myotubes B, in contrast to myotubes A, do not respond with an increase in mitochondrial ATP production upon increasing the stimulus intensity. This conclusion is in agreement with the observation that the difference in amplitude between a single stimulus and a pulse train appeared to be larger in myotubes B compared with myotubes A. Finally, the conclusion that myotubes B do have a mitochondrial memory is supported by the observation that the CRR did not change when going from the second pulse train to the last single stimulus.

Concerning myotubes A, the data presented suggest that the threshold for Ca\(^{2+}\) stimulation of the mechanism underlying the upregulation of mitochondrial ATP production is higher than in control. Therefore, this threshold is reached only during a pulse train and not during a single stimulus. In contrast, the data obtained with myotubes B suggest that the maximal degree to which Ca\(^{2+}\) can stimulate this mechanism is less than in control. It remains to be established whether these differences are also responsible for the lack of a mitochondrial memory in myotubes A and the presence of such a memory in myotubes B.

Taken together, the data presented are in agreement with a tight coupling between mitochondrial ATP production and sarcoplasmic Ca\(^{2+}\) handling. Moreover, they support the existence of a relatively long-lasting mitochondrial memory for sarcoplasmic [Ca\(^{2+}\)] handling. This memory manifested itself as an increase in sarcoplasmic Ca\(^{2+}\) removal rate upon recurrent stimulation. This upregulation of the sarcoplasmic Ca\(^{2+}\) removal rate was clearly reduced in myotubes cultured from human quadriceps muscle with a biochemically defined decrease in mitochondrial ATP production capacity. The defects in sarcoplasmic Ca\(^{2+}\) handling observed in these myotubes very well explain the muscle stiffness observed in the subjects from whom they were derived.

**REFERENCES**


15. Janssen E, Terzic A, Wieringa B, and Dzeja PP. Mitochondrial DNA. *Olson EN and Williams RS. McLennan DH.*


