Ion transport in human skeletal muscle cells: disturbances in myotonic dystrophy and Brody's disease

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INTRODUCTION

Excitation–relaxation cycle of skeletal muscle

During an action potential, the cytoplasmic Na⁺ concentration in skeletal muscle increases whereas the intracellular K⁺ concentration decreases. This depolarization of the sarcolemma provokes Ca²⁺ release from the SR into the cytoplasm, which causes muscle contraction. After excitation, ATP-driven ion pumps restore the disturbed ion homeostasis. Na⁺, K⁺ ATPase of the plasma membrane re-establishes the Na⁺, K⁺ gradients by K⁺ uptake from the blood plasma and interstitial fluid into the resting muscle and extrusion of Na⁺ (Clausen 1986). Ca²⁺ ATPase in the longitudinal part of the SR transports Ca²⁺ from the cytoplasm into the SR lumen (Carafoli 1987). In this review we will focus on these ATP-driven ion pumps, which have been shown to be affected in the muscular diseases myotonic dystrophy (Benders et al. 1993) and Brody's disease (Benders et al. 1994a), and the consequences for muscle function. Data on cultured human muscle cells are supplemented with results from physiological studies of cellular Ca²⁺ homeostasis.

Myotonic dystrophy

Myotonic dystrophy (MyD) is the most important hereditary muscle disease. Clinical manifestations...
include increased excitability, delayed relaxation, weakness and wasting of muscle, and abnormalities of various non-muscle systems (Harper 1989). Recently, the mutation underlying MyD has been discovered to be an expansion of polymorphic CTG-repeats in a gene encoding a protein kinase (myotonin protein kinase, Mt-PK; Wieringa 1994). The severity of the disease is roughly proportional to the extent of expansion of the repeat, which also explains the anticipation phenomenon, i.e. increase of severity during transmission from one generation to the next. The substrates and function of Mt-PK, however, remain unknown (Wieringa 1994).

Muscle fibres and/or cultured muscle cells of MyD patients exhibit a decreased resting membrane potential (Gruener et al. 1979, Kobayashi et al. 1990) and an increased intracellular Na⁺ concentration (Edström & Wroblewski 1989). These observations are consistent with a reduced number of ouabain-binding sites (Desnuelle et al. 1982), a raised Na⁺ conductance at rest (Rüdel & Lehmann-Horn 1985), and an altered inactivation of Na⁺ channels (Rüdel et al. 1989, Franke et al. 1990). Renaud et al. (1986) demonstrated an apamin receptor (Ca²⁺-activated K⁺ channel) in MyD muscle, which is absent in normal human muscle. Furthermore, MyD patients show an excessive plasma K⁺ increase after ischaemic exercise (Wevers et al. 1990). Moreover, we measured a raised cytosolic Ca²⁺ concentration in resting MyD muscle cells arising from abnormally active voltage-operated Ca²⁺ channels (Jacobs et al. 1991). Defective regulation of ion transport could initiate or contribute to the abnormal cellular function in MyD. We investigated the activities and contents of both Na⁺, K⁺ ATPase and SR Ca²⁺ ATPase in skeletal muscle and cultured muscle cells of MyD patients. We also examined the effect of electrochemical depolarization using 125 mm KCl on the Ca²⁺ homeostasis of cultured MyD muscle cells.

**Brady's disease**

Sarcoplasmic reticulum (SR) Ca²⁺ ATPase deficiency, i.e. Brady's disease, was first reported by Brady in 1969. The literature now cites 21 cases (Brody 1969, Karpati et al. 1986, Damon et al. 1988, Taylor et al. 1988, Benders et al. 1994a). Exercise-induced impairment of muscle relaxation, stiffening, cramps and muscle pain have been described. However, these clinical signs which correspond to pseudo-myotonia are not specific, and young patients in particular display little or no complaints (Benders et al. 1994a).

We examined the activity and content of SR Ca²⁺ ATPase in muscle and cultured muscle cells and established its deficiency in two new patients. Ca²⁺ homeostasis was studied in cultured muscle cells in order to gain an insight into the consequences of the deficiency for muscle functioning.

**MATERIALS AND METHODS**

**Patients**

Fifteen MyD patients (11 male, four female) of 12 different families varied in age between 17 and 58 years. Eleven patients were presented in a previous study (Benders et al. 1993). All patients had electromyographic evidence of myotonia, characteristic clinical features of MyD and showed an excessive K⁺ release into the blood upon a standardized ischaemic forearm exercise (Wevers et al. 1990).

In nine different families we detected 12 patients suffering from Brody's disease. Patients 1–10 have been described before (Benders et al. 1994a). Pseudo-myotonia is the most important clinical feature. By contrast with patient 12, who displayed all characterized symptoms, patient 11 showed only excercise-induced muscle pain and sometimes cramping or weakness. The latter patient is included in this study, since a positive effect of dantrolene treatment suggested an affected Ca²⁺ homeostasis, in which SR Ca²⁺ ATPase deficiency could be involved. For all patients, myotonia was excluded by electromyography. Metabolic myopathies were also excluded by appropriate investigations.

The controls displayed no metabolic or electrophysiological disorders. This study was approved by the ethical committee of the University of Nijmegen.

**Muscle cell cultures, Na⁺, K⁺ ATPase and SR Ca²⁺ ATPase**

Muscle biopsies of controls and patients were cultured as described previously (Benders et al. 1993, 1994a). Na⁺, K⁺ ATPase was investigated by measuring ouabain-binding and the activities of Na⁺, K⁺ ATPase and/or K⁺-dependent 3-O-methylfluorescein phosphatase (3-OMFPase; Benders et al. 1992). SR Ca²⁺ ATPase was examined by Ca²⁺-dependent phosphorylation and its activity on ATP in the presence of 5 μm free Ca²⁺ (Benders et al. 1992).

**Ca²⁺ homeostasis**

The free cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined in cultured muscle cells using Fura-2 ratio measurement (Benders et al. 1994a, Grynkiewicz et
**RESULTS**

**Muscle characteristics**

The non-collagen protein content is comparable in quadriceps muscle of both controls and patients suffering from MyD or Brody’s disease and varies between 130 and 160 mg (g wet wt)⁻¹. The fibre type composition is also similar (34–51% type 1 fibres).

**Immunocytochemistry**

The sarcolemma of both fast- and slow-twitch muscle fibres are strongly positive when stained for Na⁺, K⁺ ATPase. In longitudinal sections, cross-striation is visible at the I-band. Cultured myotubes are also immunoreactive, but cross-striation at the I-band is only observed in highly matured muscle cells.

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*Figure 1* The activities of K⁺-dependent 3-O-MFPase (a) and Na⁺, K⁺ ATPase (c), and the ouabain-binding capacities (b, d) in relation to age and maturation grade of muscle (a, b) and cultured muscle cells (c, d), respectively, for individual controls (○) and MyD patients (■). The solid lines represent the linear regression curves of the control values (P < 0.01) and the dotted lines indicate the 99% confidence intervals (adapted from Benders et al., 1993, with permission).

Data represent means ± SD. Statistical analysis was performed by means of the unpaired Student’s t-test and significance was set at P < 0.01.

**Other procedures**

As a measure of the maturation grade of muscle cells, the percentage of creatine kinase MM (CK-MM) was used (Benders *et al*. 1992). For fibre type analysis, myofibrillar ATPase was histochemically investigated. Immunocytochemistry on Na⁺, K⁺ ATPase and SR Ca²⁺ ATPase was performed using the methods described previously (Benders *et al*. 1992, 1994a).
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Figure 2 The SR Ca²⁺-ATPase activities (a, c) and concentrations (b, d) in relation to ageing and maturation grade of muscle (a, b) and cultured muscle cells (c, d), respectively, for individual controls (O) and MyD patients (■). For other details see the legend to Figure 1 (adapted from Benders et al. 1993, with permission).

Myotonic dystrophy: Na⁺, K⁺-ATPase and SR Ca²⁺-ATPase

Na⁺, K⁺-ATPase activity is hardly detectable in homogenates of muscle biopsies, because of an excess of Mg²⁺ ATPase activity. The K⁺-dependent hydrolytic cleavage of the artificial substrate 3-O-MFP and its inhibition by ouabain can be used as a measure for Na⁺, K⁺ ATPase activity in muscle (Norgaard et al. 1984). Ageing (0-65 years) had no effect on the K⁺-dependent 3-O-MFPase activity or the maximal ouabain binding capacity, i.e. the number of Na⁺, K⁺-ATPase molecules (Figs 1a & b). The Na⁺, K⁺ ATPase content of normal human skeletal muscle (360 ± 70 pmol (g wet wt)⁻¹, n = 5) agrees with earlier data (Klitgaard & Clausen 1989, Kjeldsen et al. 1990). The latent Na⁺, K⁺ ATPase activity is maximally exposed in freeze-thawed homogenates of cultured cells (Benders et al. 1992). The Na⁺, K⁺ ATPase and K⁺-dependent 3-O-MFPase activities and the number of Na⁺, K⁺ ATPase molecules increase in cultured muscle cells when their maturation grade increases (Figs 1c & d) and are always higher than in muscle biopsies. The dissociation constant of ouabain (240 nm) and the molecular 3-O-MFPase activity (100 min⁻¹) do not differ in skeletal muscle and cultured muscle cells.

In skeletal muscle of MyD patients, both the K⁺-dependent 3-O-MFPase activity and the ouabain-binding capacity are lowered by 35-40% (Figs 1a & b). In cultured muscle cells of MyD patients the Na⁺, K⁺ ATPase activity and the number of ouabain-binding sites are significantly reduced (35-40%) compared with control cells of the same maturation grade (Figs 1c & d). The dissociation constant of

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ouabain and the molecular activity do not differ in skeletal muscle and cultured muscle cells of controls and MyD patients. These results imply that the decrease of the Na\(^+\), K\(^+\) ATPase activity in MyD is caused by a reduced concentration of Na\(^+\), K\(^+\) ATPase.

SR Ca\(^{2+}\) ATPase is maximally activated at a free Ca\(^{2+}\) concentration of about 5 \(\mu\)M and thapsigargin (1 \(\mu\)M) inhibits 90% of the Ca\(^{2+}\)-dependent ATPase activity and phosphorylation in muscle and cultured muscle cells (Benders et al., 1992). Thus the measured parameters represent quite well the parameters of the SR Ca\(^{2+}\) pump. The content of SR Ca\(^{2+}\) ATPase can be quantified by Ca\(^{2+}\)-dependent phosphorylation under steady-state conditions (Everts et al., 1992). The activity and concentration of SR Ca\(^{2+}\) ATPase show a slight but significant age-related decrease in human muscle (Figs 2a & b). The content of SR Ca\(^{2+}\) ATPase of adult human skeletal muscle (6.7 ± 1.1 nmol (g wet wt) \(^{-1}\), \(n = 29\)) is comparable to previously published data (Everts et al., 1992). Both the activity and content of SR Ca\(^{2+}\) ATPase rise in cultured muscle cells with the maturation grade (Figs 2c & d), but remain lower than in muscle biopsies. The molecular activity of SR Ca\(^{2+}\) ATPase (800 min \(^{-1}\)) is similar in muscle and cultured muscle cells.

The activity and the content of SR Ca\(^{2+}\) ATPase are reduced by 35-40% in both muscle and cultured muscle cells of MyD patients, as compared with controls of the same age and maturation grade, respectively (Fig. 2). The molecular activity of SR Ca\(^{2+}\) ATPase does not differ from the control values. These observations indicate that the decrease of SR Ca\(^{2+}\)-ATPase activity in MyD is due to a reduction of the SR Ca\(^{2+}\) ATPase content.

Myotonic dystrophy: [Ca\(^{2+}\)]\(_i\) and the effects of KCl and nifedipine

The basal [Ca\(^{2+}\)]\(_i\) in cultured muscle cells of controls is 136 ± 11 nM (\(n = 12\)). Electrochemical depolarization of the sarcolemma using 125 mM KCl increases [Ca\(^{2+}\)]\(_i\) to 1.24 ± 0.25 \(\mu\)M in the presence as well as in the absence of extracellular Ca\(^{2+}\) (Fig. 3a). The half-times \(\tau_f\) and \(\tau_m\) are 15 ± 5 s and 21 ± 6 s, respectively. The KCl-induced Ca\(^{2+}\) transient can be inhibited by 10 \(\mu\)M nifedipine (Fig. 3b).
In muscle cells of MyD patients the basal $[\text{Ca}^{2+}]_i$ is significantly higher (197 ± 20 nm, $n = 6$) but the $\text{Ca}^{2+}$ response upon stimulation with 125 mM KCl is less (increase to 0.91 ± 0.13 μM; $P < 0.01$) and slower ($\tau_r$ is 22 ± 4 s; $P < 0.01$) (Fig. 3c). The restoration of the basal $[\text{Ca}^{2+}]_i$ is unaffected ($\tau_r$ is 25 ± 5 s). The addition of 10 μM nifedipine prior to KCl stimulation decreases $[\text{Ca}^{2+}]_i$ to 142 ± 18 nm ($n = 4$), a value comparable to the basal $[\text{Ca}^{2+}]_i$ of control muscle cells. Subsequently, addition of KCl does not affect $[\text{Ca}^{2+}]_i$ (Fig. 3d). These results imply that the elevated basal $[\text{Ca}^{2+}]_i$ in MyD muscle cells is due to active voltage-operated $\text{Ca}^{2+}$ channels, which are inactive in resting muscle cells of controls. Besides, these channels were also unable to respond to depolarization caused by 100 μM acetylcholine (ACh); (Jacobs et al. 1991).

**Brody’s disease; SR $\text{Ca}^{2+}$ ATPase, SERCA isoforms and Na+, K+ ATPase**

The total SR $\text{Ca}^{2+}$-ATPase content was measured by ELISA with a polyclonal antibody, raised against a mixture of both fast- and slow-type SR $\text{Ca}^{2+}$ ATPase. On the basis of the molecular mass (110 kDa) the results confirm the data obtained by phosphorylation. Since in humans the content of SR $\text{Ca}^{2+}$ ATPase is higher in fast-twitch than in slow-twitch muscle fibres (Benders et al. 1992) and the isoforms are different for both fibre types (Brandi et al. 1986), the contribution of the fast-twitch muscle isoform (SERCA1) to the total SR $\text{Ca}^{2+}$ ATPase content was investigated. SERCA1, quantified by a specific monoclonal antibody, contributes 83 and 100%, respectively, to the total SR $\text{Ca}^{2+}$ ATPase concentrations in quadriceps muscle and cultured muscle cells.

The activity of SR $\text{Ca}^{2+}$ ATPase is reduced by 50.7 ± 7.4% in muscle and 61.2 ± 7.2% in cultured muscle cells of the patients suffering from Brody’s disease, as compared with controls of the same age and maturation grade, respectively (Figs 4a & c). However, the concentration of SR $\text{Ca}^{2+}$ ATPase, measured by phosphorylation, is not affected either in muscle or in cultured muscle cells of the patients (Figs 4b & d). Besides, the concentration of the SERCA1...
Figure 5 The $\text{Ca}^{2+}$ response in muscle cells of controls (dotted traces) and patients suffering from Brody's disease (solid traces) upon the successive addition of ACh and dantrolene (a) or verapamil (b), or the addition of ACh in the presence of dantrolene (c) or verapamil (d). Adapted from Benders et al. (1994a), with permission.

Isoform is also normal in both muscle and cultured cells of the patients. Consequently, the molecular activity of the SERCA1 isoform is about 50% lower in the pathological muscle.

In muscle and cultured muscle cells of the patients, the activities of K+-dependent 3-O-MFPase, as a measure of Na+, K+ ATPase activity, are similar to controls of the same age (0.41 U (mg protein)$^{-1}$) and maturation grade (1.11 U (mg protein)$^{-1}$), respectively.

DISCUSSION

Na+, K+ ATPase and SR Ca2+ ATPase in skeletal muscle and cultured muscle cells

A tight control of the Na+, K+ transport is essential for the maintenance of optimal muscle function (Clausen 1986). The capacity for muscle performance is evidently related to the Na+, K+-ATPase con-
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Centration (Kjeldsen et al. 1988). Our observation on the age-independent 3-O-MFPase activity and ouabain-binding capacity is in agreement with the absence of changes in the number of Na\textsuperscript{+}, K\textsuperscript{-}-ATPase molecules in skeletal muscle between 0 and 86 years (Dorup et al. 1988, Kjeldsen & Gron 1989, Klitgaard & Clausen 1989). The similar immunocytochemical staining for Na\textsuperscript{+}, K\textsuperscript{-} ATPase in human fast- and slow-twitch fibres indicates that there are no marked differences of ion pump density between both fibre types. The higher values of Na\textsuperscript{+}, K\textsuperscript{-} pump parameters in cultured human muscle cells compared with adult skeletal muscle and their increase during maturation may reflect a preferential synthesis of the T-tubule proteins such as Na\textsuperscript{+}, K\textsuperscript{-} ATPase in the first phase of muscle growth (Benders et al. 1992).

SR Ca\textsuperscript{2+} ATPase constitutes about 90% of the total protein content in the SR membrane (Fleischer & Inui 1989). The SR Ca\textsuperscript{2+} ATPase content is about six times higher in fast-twitch than in slow-twitch fibres (Benders et al. 1994a). The decrease in the activity and concentration of SR Ca\textsuperscript{2+} ATPase in human skeletal muscle upon ageing is associated with a selective atrophy of fast-twitch fibres (Poggi et al. 1987, Klitgaard et al. 1989). The rise of SR Ca\textsuperscript{2+} ATPase parameters during maturation may explain the progressive decrease in the basal [Ca\textsuperscript{2+}]\textsubscript{i} in differentiating myotubes (Cognard et al. 1993) and may relate to the development of contractile activity.

Immunocytochemical localization of Na\textsuperscript{+}, K\textsuperscript{-} ATPase and SR Ca\textsuperscript{2+} ATPase in highly matured cultured muscle cells reveals a cross-striated appearance, indicating the development of a T-tubule system and the subcellular organization of the SR, which resembles the in vivo situation (Benders et al. 1992).

Ca\textsuperscript{2+} homeostasis in cultured skeletal muscle cells

We were unable to study Ca\textsuperscript{2+} homeostasis in separate fibres from human muscle, because it is impossible to isolate them intact. Isolated mononuclear satellite cells are not suitable, since these undifferentiated cells lack a signal transduction system, which resembles the in vivo situation (Cognard et al. 1993). The basal [Ca\textsuperscript{2+}]\textsubscript{i} in cultured muscle cells of controls is comparable with published data (Sarabia & Klip 1989, Jacobs et al. 1991). Since the maximal [Ca\textsuperscript{2+}]\textsubscript{i} values after stimulation with KCl or ACh are equal both in the presence and absence of extracellular Ca\textsuperscript{2+}, the rise in cytosolic Ca\textsuperscript{2+} originates from the Ca\textsuperscript{2+} release of the SR. This indicates a 'skeletal' type of excitation-contraction coupling in our cultured human skeletal muscle cells rather than a 'cardiac' type (Cognard et al. 1992). Two oppositely acting mechanisms determine the shape of the Ca\textsuperscript{2+} transient. On the one hand, in the presence of KCl or ACh the plasma membrane is depolarized and the dihydropyridine receptor/ryanodine receptor (DHPR-RyR) complex mediates the Ca\textsuperscript{2+} release from the SR. On the other hand, the SR Ca\textsuperscript{2+} pump is activated by the increased cytosolic Ca\textsuperscript{2+} concentration and restores the [Ca\textsuperscript{2+}]\textsubscript{i}.

Myotonic dystrophy

Myotonia is a transient uncontrollable muscle tension during muscle contraction, which is caused by trains of repetitive action potentials in response to a contraction, and as a result the skeletal muscles are unable to relax normally (Rüdel & Lehmann-Horn 1985). All myotonias appear to be due to an abnormality of the muscle itself, since they persist after section or blocking of the motor nerve and after curarization (Carafoli 1987). Several mechanisms have been proposed to explain the different types of myotonia. A reduced chloride conductance underlies myotonia congenita (Koch et al. 1992); however, it seems to be insignificant in MyD (Rüdel & Lehmann-Horn 1985). In primary hyperkalaemic periodic paralysis and MyD, an increased permeability to Na\textsuperscript{+} appears to be responsible for the myotonia (Rüdel 1986). Defects in the α-subunit of the human skeletal muscle sodium channel, due to mutations, result in the former disorder and in paramyotonia congenita and potassium-aggravated myotonia (Hoffman et al. 1995). In MyD, the product of the abnormal gene is not a protein subunit of an ion channel, but a protein kinase, called myotonin protein kinase (Mt-PK). This kinase appears to modulate skeletal muscle sodium channels, when co-expressed in Xenopus oocytes, by reducing the amplitude of the Na\textsuperscript{+} currents and accelerating the current decay (Mounsey et al. 1995). However, the mutation that was responsible for the disease-trinucleotide repeats in the 3' untranslated region did not prevent the effect. The relation between the mutation and the deficiency of Na\textsuperscript{+}, K\textsuperscript{-} ATPase and SR Ca\textsuperscript{2+} ATPase deserves investigation.

The reduction of Na\textsuperscript{+}, K\textsuperscript{-} ATPase activity as a result of the decreased Na\textsuperscript{+}, K\textsuperscript{-} ATPase content in muscle and cultured muscle cells of MyD patients can explain the raised intracellular Na\textsuperscript{+} concentration (Eldström & Wrobleski 1989) and the lower resting membrane potential (Gruener et al. 1979, Kishiyashishi et al. 1990). Furthermore, it might contribute to the abnormally high increase of plasma K\textsuperscript{-} concentration in MyD patients upon muscular exercise, and the reduced amount of work performed by these patients (Wevers et al. 1990).
In muscle and cultured cells of MyD patients, the decrease in the activity of the SR Ca\(^{2+}\) ATPase is caused by the reduction of the SR Ca\(^{2+}\)-ATPase protein content. This reduction of Ca\(^{2+}\) pumps in the SR may contribute to the abnormal relaxation of MyD muscle and reinforce the myotonia. Moreover, in combination with the presence of an additional type of Ca\(^{2+}\)-activated K\(^{+}\) channel (apamin receptor (Renaud et al. 1986)), it can cause the excessive increase of plasma K\(^{+}\) (Wevers et al. 1990), which in turn can induce myotonia (Appel et al. 1984).

The reductions in the concentrations of both ion pumps investigated are of the same order of magnitude in both the muscle and cultured muscle cells of MyD patients. The differences seem to be specific, because neither the fibre type distribution nor the protein content of the examined MyD muscle is changed compared with the control muscle. Fibre type I predominance was not observed in MyD muscle in contrast to previously published data (Borg et al. 1987). The activities and content of Na\(^{+}\), K\(^{+}\) ATPase and SR Ca\(^{2+}\) ATPase are of the same order in both highly maturated cultured MyD muscle cells and lowly maturated control cells. A differentiation-related disturbance of membranes might be the origin of the measured alterations in muscle of MyD patients. This is also suggested by morphological observations in neonatal MyD muscle (Sarnat & Silbert 1976) and by the persistent expression of two membrane antigens (Walsh et al. 1988) and the apamin receptor (Renaud et al. 1986) in MyD muscle.

Cultured MyD muscle cells exhibit a higher basal [Ca\(^{2+}\)] due to changed dihydropyridine receptors (DHPRs), i.e. voltage-operated Ca\(^{2+}\) channels, which are already active at rest, since the increased basal [Ca\(^{2+}\)], normalized completely upon the addition of nifedipine, a specific inhibitor of DHPR. Nifedipine treatment also suppressed myotonia in patients (Grant et al. 1987). One of the functions of Mr-PK as described by Mounsey et al. (1995) is phosphorylation of an inactivation site of the skeletal muscle sodium channel. Impaired ion channel regulation could explain the abnormal activity/functioning of the DHPRs. ACH (100 µM) was unable to increase [Ca\(^{2+}\)] in cultured MyD muscle cells, although the sarcolemma was effectively depolarized (Jacobs et al. 1991). Electrochemical depolarization of cultured muscle cells using 125 mM KCl releases Ca\(^{2+}\) from SR into the cytoplasm by activating the DHPR–RyR complex. MyD muscle cells, however, are less excitable than control cells because the Ca\(^{2+}\) response upon KCl stimulation is smaller and slower. These effects aggravate during repetitive KCl stimulation (Benders et al., unpubl. data). The smaller and slower KCl-induced Ca\(^{2+}\) transient explains the muscle weakness in MyD, but opposes myotonia and may act as a safety mechanism. In addition, despite the reduction of their SR Ca\(^{2+}\)-ATPase activity and content by 35-40%, cultured muscle cells of MyD patients are able to recover from a KCl-induced Ca\(^{2+}\) transient as quickly as do control cells. This suggests that control cells have a functional redundancy of SR Ca\(^{2+}\) ATPase.

**Brody's disease**

Since our recent paper (Benders et al. 1994a), we have detected two new patients (11 and 12). The signs and symptoms of the patients are rather heterogeneous and non-specific. Even impaired muscle relaxation can be absent in some cases, as in our patients 1, 2, 5, 9 and 11 and in one patient described by Taylor et al. (1988). The diagnosis, Brody's disease, should be considered more often in patients with exercise-induced muscle stiffness, cramp and pain syndromes.

Various authors have used a wide range of techniques to assay SR Ca\(^{2+}\) ATPase in human muscle. In only two of the 11 patients presented in the literature (by others) were the activity and concentration of the SR Ca\(^{2+}\) pump biochemically characterized (Karpati et al. 1986, Taylor et al. 1988). The SR Ca\(^{2+}\)-ATPase activity or ATP-dependent Ca\(^{2+}\) uptake determined with microsomal or SR fractions varied between 2 and 30% of the control values (Brody 1969, Karpati et al. 1986, Taylor et al. 1988). Since the preparation of microsomal or SR fractions may introduce differences in recovery, the assay of SR Ca\(^{2+}\)-ATPase activity and protein content can be better performed on whole muscle homogenates (Ruell et al. 1995). From our phosphorylation and ELISA assays and cytochemical staining, it appeared that, in both the muscle and cultured muscle cells of our patients, the decrease in SR Ca\(^{2+}\)-ATPase activity is not caused by a reduction of the SR Ca\(^{2+}\)-ATPase protein content. This agrees with the results of Taylor et al. (1988), but not with other data in the literature (Karpati et al. 1986, Danon et al. 1988).

The high contributions of SERCA1 to the total SR Ca\(^{2+}\)-ATPase content are comparable in both controls and patients. This implies that in Brody's disease the molecular activity of the SERCA1 isoform is reduced. The isoforms SERCA1 and SERCA2a show similar enzyme characteristics (Lytton et al. 1992). An isoform switch of SERCA1 to SERCA2a can be excluded by the results obtained from cultured muscle cells, in which 100% of the SERCA1 is present.

In Brody's disease, the disturbance of the SR Ca\(^{2+}\) pump seems to be specific, since the fibre-type distribution, the total protein content and the Na\(^{+}\), K\(^{+}\)-ATPase activity are normal. Substitutions of certain
amino acids by site-directed mutagenesis in the stalk, hinge or transmembrane domains of SR Ca\textsuperscript{2+} ATPase cause a marked reduction of Ca\textsuperscript{2+} transport and ATPase activity without affecting phosphorylation (Vilsen et al. 1991a, b, Andersen et al. 1992). Other mutations inhibit phosphorylation of the SR Ca\textsuperscript{2+} pump or uncouple hydrolysis from Ca\textsuperscript{2+} transport (Andersen 1995). It remains to be elucidated whether, and how, the SERCA1 gene on chromosome 16 (MacLennan et al. 1987) is involved in Brody's disease.

Since the deficiency is expressed in the same way in both muscle and cultured muscle cells, it was possible to examine the Ca\textsuperscript{2+} homeostasis. In addition, this \textit{in vitro} model was used to investigate the effects of potential drugs. Since the maximal \([\text{Ca}^\text{2+}]_i\), after ACh stimulation and the half-time of \([\text{Ca}^\text{2+}]_i\) increase (\(\tau_i\)) are the same in muscle cells of controls and patients with Brody's disease, the depolarization of the muscle cell membrane by ACh and the subsequent Ca\textsuperscript{2+} release from the SR are normal in the diseased muscle cells. This is in agreement with the \textit{in vivo} situation. In electromyography, patients show a physiological recruitment of normal motor unit potentials (Brody 1969, Karpati et al. 1986, Taylor et al. 1988, Wevers et al. 1992). In the muscle cells of the patients, the increased time needed for Ca\textsuperscript{2+} reuptake from the cytosol after excitation is a consequence of the decreased activity of the SR Ca\textsuperscript{2+} pump. This may result in pseudo-myotonia \textit{in vivo}. At addition of dantrolene or verapamil, which are blockers of the DHPR–RyR complex (Valdivia et al. 1990, El-Hayek et al. 1992), the release of Ca\textsuperscript{2+} is inhibited, but the SR Ca\textsuperscript{2+} pump is still active and the restoration of the basal \([\text{Ca}^\text{2+}]_i\) becomes accelerated. It is unlikely that, at this point (\(\langle \text{Ca}^\text{2+}\rangle_i \approx 200 \text{ m}\)), other Ca\textsuperscript{2+}-restoring processes, e.g. the sarcosomal Ca\textsuperscript{2+} ATPase and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, are involved in the restoration of the basal \([\text{Ca}^\text{2+}]_i\).

The sarcosomal Ca\textsuperscript{2+} pump amounts to only 10% of the total Ca\textsuperscript{2+}-dependent ATPase activity in control cells (Benders et al. 1992) and probably to about 20% in the muscle cells of the patients as a result of the 50% decreased SR Ca\textsuperscript{2+}-ATPase activity. Moreover, the sarcosomal Ca\textsuperscript{2+} ATPase has a lower affinity for \([\text{Ca}^\text{2+}]_i\) than does the SR Ca\textsuperscript{2+} pump (Carafoli 1992). On the basis of a \([\text{Ca}^\text{2+}]_i\) of 200 m\textsuperscript{+} and a cytosolic Na\textsuperscript{+} concentration of 12.4 m\textsuperscript{+} (Benders et al. 1994b), calculations presented previously show that the Na\textsuperscript{+}, Ca\textsuperscript{2+} electrochemical potential gradients are balanced, which means that the Na\textsuperscript{+}, Ca\textsuperscript{2+} exchanger is not functioning.

Upon simultaneous addition of ACh and either dantrolene or verapamil, the Ca\textsuperscript{2+} response is reduced. Due to the lower maximal \([\text{Ca}^\text{2+}]_i\), SR Ca\textsuperscript{2+} ATPase is less activated and the half-time of the Ca\textsuperscript{2+} decay (\(\tau_i\)) is increased in control cells (Benders et al. 1992). However, since less Ca\textsuperscript{2+} pumping capacity is required to restore \([\text{Ca}^\text{2+}]_i\), the \(\tau_i\) values become equal in cultured muscle cells of both controls and patients. So, if a Ca\textsuperscript{2+} overload is prevented in the diseased cells, they seem to be able to handle the Ca\textsuperscript{2+} response as do control cells. These observations may explain the beneficial effect of administration of dantrolene (Wevers et al. 1992, Poels et al. 1993) or verapamil (Taylor et al. 1988) to patients suffering from Brody's disease.

**GENERAL CONCLUSIONS**

Cultured human muscle cells may well be applied in studies of ion transport and homeostasis during myogenesis and muscle diseases. Cultured muscle cells from patients suffering from MyD or Brody's disease reflect muscle characteristics well.

In skeletal muscle and cultured muscle cells from MyD patients, the decrease in the activities of Na\textsuperscript{+}, K\textsuperscript{+} ATPase and SR Ca\textsuperscript{2+} ATPase is due to a reduction of their enzyme protein contents. A differentiation-related disturbance of membranes or a modulation defect of membrane proteins may play a role in MyD. Furthermore, cultured MyD muscle cells possess abnormally active DHPRs and as a result exhibit an increased \([\text{Ca}^\text{2+}]_i\) at rest. The detected changes may contribute to the characteristics of the muscle pathology in MyD.

In muscle of patients suffering from Brody's disease, the activity (but not the concentration) of the predominant SERCA1 isoform of SR Ca\textsuperscript{2+} ATPase is decreased. Structural modifications of SERCA1 may be involved. The delayed restoration of the cytosolic Ca\textsuperscript{2+} concentration after excitation may explain the impaired muscle relaxation, stiffness and cramping. Reduction of the Ca\textsuperscript{2+} release by dantrolene or verapamil balances the excitation–relaxation cycle in these pathological cells.

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