Ca²⁺ Homeostasis in Brody's Disease

A Study in Skeletal Muscle and Cultured Muscle Cells and the Effects of Dantrolene and Verapamil

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Abstract

Brody's disease, i.e., sarcoplasmic reticulum (SR) Ca²⁺-dependent Mg²⁺-ATPase (Ca²⁺-ATPase) deficiency, is a rare inherited disorder of skeletal muscle function. Pseudo-myotonia is the most important clinical feature. SR Ca²⁺-ATPase and Ca²⁺ homeostasis are examined in m. quadriceps and/or cultured muscle cells of controls and 10 patients suffering from Brody's disease.

In both m. quadriceps and cultured muscle cells of patients, the SR Ca²⁺-ATPase activity is decreased by ~ 50%. However, the concentration of SR Ca²⁺-ATPase and SERCA1 are normal. SERCA1 accounts for 83 and 100% of total SR Ca²⁺-ATPase in m. quadriceps and cultured muscle cells, respectively. This implies a reduction of the molecular activity of SERCA1 in Brody's disease.

The cytosolic Ca²⁺ concentration ($[Ca^{2+}]_i$) at rest and the increase of $[Ca^{2+}]_i$ after addition of acetylcholine are the same in cultured muscle cells of controls and patients. The half-life of the maximal response, however, is raised three times in the pathological muscle cells. Addition of dantrolene or verapamil after the maximal response accelerates the restoration of the $[Ca^{2+}]_i$ in these muscle cells. The differences in Ca²⁺ handling disappear by administration of dantrolene or verapamil concomitantly with acetylcholine.

The reduced Ca²⁺ re-uptake from the cytosol presumably due to structural modification(s) of SERCA1 may explain the pseudo-myotonia in Brody's disease. Single cell measurements suggest a beneficial effect of dantrolene or verapamil in treating patients suffering from Brody's disease. (*J. Clin. Invest.* 1994. 94:741–748.) Key words: Brody's disease • sarcoplasmic reticulum Ca²⁺-ATPase deficiency • Ca²⁺ homeostasis • dantrolene • verapamil

Introduction

Sarcoplasmic reticulum (SR)¹ Ca²⁺-ATPase deficiency, Brody's disease (McKusick 108740) has for the first time been observed by Brody in 1969 (1). In literature 12 cases have been reported (1–6). The clinical signs and symptoms in patients suffering from Brody's disease are not specific. Exercise-induced impairment of muscle relaxation (1), stiffening and cramps (2, 3) and muscle pain (4) have been described. These symptoms may exacerbate in the cold (4). The clinical features correspond to pseudo-myotonia.

Defective regulation of ion transport can initiate or contribute to the abnormal cellular function in Brody's disease (1, 2, 4). In muscle, an action potential is associated with an influx of Na⁺ and an efflux of K⁺. This depolarization of the sarcolemma induces a Ca²⁺ release from the SR into the cytosol which causes muscle contraction. After excitation of skeletal muscle, ATP-dependent ion pumps restore the disturbed ion homeostasis. SR Ca²⁺-ATPase transports Ca²⁺ from the cytosol into the lumen of SR (7) and Na⁺ + K⁺-dependent ATPase (Na⁺/K⁺-ATPase) re-uptakes K⁺ from the interstitial fluid into the muscle and releases Na⁺ (8).

Previously we studied various parameters of SR Ca²⁺-ATPase and Na⁺/K⁺-ATPase in human skeletal muscle and cultured human muscle cells (9). In highly differentiated cultured muscle cells we also observed the development and subcellular organization of a T-tubule system and SR, which resemble the in vivo situation (9). For a correct diagnosis of Brody's disease, investigation of the SR Ca²⁺-pump in muscle biopsy is required, since a simple diagnostic test is not yet available. In this study we examined the activity and content of SR Ca²⁺-ATPase in muscle homogenates and cultured muscle cells and established its deficiency in 10 patients. Since the content of SR Ca²⁺-ATPase is higher in type II muscle fibers than in type I fibers (9) and the isoforms are different for both fiber types (10), the contribution of the fast-twitch muscle SR Ca²⁺-ATPase isoform (SERCA1) to the total SR Ca²⁺-ATPase content was investigated. Na⁺/K⁺-ATPase was examined to check whether the SR Ca²⁺-ATPase is specifically affected or a common defect in all ATP-dependent ion pumps is the basis of Brody's disease. Ca²⁺ homeostasis was studied in cultured muscle cells to gain an insight into the consequences of the deficiency for cell functioning. In addition, we used the muscle cells to study the potential beneficial effects of dantrolene and verapamil on Ca²⁺ homeostasis.

Methods

Materials. Fura-2/acetoxymethyl ester (Fura-2/AM), acetylcholine chloride (ACh) and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO), dantrolene from Norwich Eaton, (Rotterdam, The

1. *Abbreviations used in this paper:* ACh, acetylcholine; Ca²⁺-ATPase, Ca²⁺-dependent Mg²⁺-ATPase (EC 3.6.1.38); $[Ca^{2+}]_i$, cytosolic free Ca²⁺ concentration; CK-MM, creatine kinase muscle-specific isoenzyme MM (EC 2.7.3.2); Fura-2/AM, Fura-2/acetoxymethyl ester; 3-O-MFPase, 3-O-methylflourescein phosphatase; Na⁺/K⁺-ATPase, Na⁺ + K⁺-dependent ATPase (EC 3.6.1.37); PSS, physiological salt solution; SERCA1, SR Ca²⁺-ATPase isoform of fast-twitch muscle; SR, sarcoplasmic reticulum.

Table I. Exercise-induced Symptoms in Patients Suffering from Brody's Disease

Patient	Sex	Age	Impaired relaxation	Stiffness	Cramping	Weakness	Pain
1	F	10	−	−	−	+	+
2	M	14	−	−	−	+	+
3	M	15	+	+	+	+	+
4	M	16	+	+	+	+	+
5	F	17	−	+	−	−	−
6	M	29	+	+	+	+	+
7	F	29	+	+	+	−	+
8	F	30	+	+	+	+	+
9	M	36	−	+	+	+	+
10	F	55	+	+	+	+	+

Symptoms which are present (+) or absent (−) in the individual patients. Besides, patients 7 and 9 displayed many episodes of rhabdomyolysis.

Netherlands), and Isoptin®, i.e., verapamil hydrochloride, from Knoll BV, (Amsterdam, The Netherlands). The monoclonal antibody 5D2, i.e., mouse anti–chicken fast-twitch muscle SR Ca^{2+}-ATPase (SERCA1) was obtained from Dr. D. M. Fambrough and was originally prepared by Kaprielian and Fambrough (11). It is present as CaF2-5D2 in the Developmental Studies Hybridoma Bank (The Johns Hopkins University, Baltimore, MD). The polyclonal antibody, i.e., rabbit anti–rat SR Ca^{2+}-ATPase, was a gift of Dr. J. Timmermans. This antibody was raised against a mixture of slow- and fast-type SR Ca^{2+}-ATPase. Other materials were obtained as described previously (9).

Patients. In seven different families we detected 10 patients suffering from Brody's disease. The symptoms of the individual patients are presented in Table I. Patient 1 is a sister of patient 2, patient 3 and 4 are brothers, whereas patient 6 is a son of patient 10. Pseudo-myotonia is the most important clinical feature. Patients 1, 2, and 9 are included in this study, since a positive effect of dantrolene treatment suggested an affected Ca^{2+} homeostasis, in which SR Ca^{2+}-ATPase deficiency could be involved. Patient 9 was previously described (5, 6). Patient 5 presented fatigue symptoms and biochemical examinations revealed the SR Ca^{2+}-ATPase deficiency. For all patients, myotonia was excluded by electromyography. Glycolytic, mitochondrial and lipid storage myopathies were also excluded by appropriate investigations.

Muscle cell cultures and homogenates. Biopsies from m. quadriceps of controls and patients 2, 4, 5, and 6 were dissociated according to Yasin et al. (12). For measurements of ATP-driven ion pumps, muscle cells were cultured on sera- or Ultroser G-containing media and homogenates were prepared as previously described (9). Cell physiological studies were performed with muscle cells grown on glass coverslips (∅ 22 mm) which proliferated and differentiated on sera-containing media (13).

SR Ca^{2+}-ATPase and Na^+/K^+-ATPase. The activity and concentration of SR Ca^{2+}-ATPase were investigated by measuring Ca^{2+}-dependent ATP hydrolysis and steady state phosphorylation, respectively (9). Moreover, total SR Ca^{2+}-ATPase and SERCA1 were quantified by using a polyclonal and a specific monoclonal (5D2) antibody, respectively, in ELISA as described previously (9). The activity of Na^+/K^+-ATPase was measured as K^+-dependent and ouabain-sensitive 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (9).

Ca^{2+} homeostasis: on-line ratio measurement and digital imaging. The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined in single cells using Fura-2 ratio measurement (14) and digital imaging (15). Myotubes were washed with physiological salt solution (PSS; containing in mM: 125 NaCl, 10 NaHCO₃, 1 NaH₂PO₄, 5 KCl, 2 MgSO₄, 1.8 CaCl₂, 10 Hepes and 10 glucose, pH 7.4) and loaded with 5 µM Fura-2/AM in PSS for 60 min at 37°C. Excess of dye was removed by washing thrice with PSS. Coverslips were mounted into a

heating chamber (37°C). A Nikon Diaphot epifluorescence microscope was used to focus the cells onto a photomultiplier tube, for on-line ratio measurement, or a charge-coupled device camera (Photonics Science, Robertsbridge, U. K.) for digital imaging. The excitation light was passed alternately through filters of 340 and 380 nm (10 and 13 nm bandwidths, respectively) and the emission light was collected through a 400-nm dichroic mirror and a 492-nm bandpass filter. Ratio measurements were performed using the NPS Photon Counting System (Newcastle Photometric Systems, Newcastle, U. K.). For digital imaging the MagiCal hardware and TARDIS software of Joyce-Loebl (Gateshead, U. K.) were utilized. Video images were hardware averaged 8 times, to reduce noise of the camera. The 340:380 ratio was calculated on a pixel-by-pixel basis. All fluorescence signals were corrected for background and autofluorescence. During the measurements the cells were superfused with PSS (2.5 ml·min⁻¹; 37°C) with additions as indicated in the results. The signals were calibrated with 4 µM ionomycin in the presence of 10 mM Ca^{2+} and with 20 mM EGTA, pH 8.5.

Other procedures. For the determination of the percentage creatine kinase MM (CK-MM), as measure of the maturation grade of muscle cells, this isoenzyme was separated from the other CK isoenzymes by anion exchange chromatography and analysed as reported (16). The protein content was assayed according to Lowry et al. (17) with bovine serum albumin as standard. For fiber type analysis, myofibrillar ATPase was histochemically investigated (18). Immunocytochemistry on SR Ca^{2+}-ATPase and SERCA1 was performed according to the method described previously (9).

Statistics. All experiments were performed in duplicate. Moreover, the enzyme measurements in the pathological muscle samples were done in two separate experiments. Data represent means±SD. Statistical analysis was performed by means of the unpaired Student's t test and significancy was set at $P < 0.01$.

Results

General characteristics of muscle. The non-collagen protein content is comparable in m. quadriceps of controls and patients and varies between 130 and 160 mg/g wet wt. The fiber type composition is also similar (34–51% type I fibers).

SR Ca^{2+}-ATPase. SR Ca^{2+}-ATPase is maximally activated at a free Ca^{2+} concentration of 5 µM and thapsigargin (1 µM), a specific inhibitor of the ion pump, suppresses 90% of the Ca^{2+}-dependent ATPase activity and phosphorylation (9). Thus the measured parameters characterize the SR Ca^{2+}-ATPase quite well. The total concentration of SR Ca^{2+}-ATPase was quantified by Ca^{2+}-dependent phosphorylation under steady state conditions, since all enzyme molecules are in a phosphorylated state and dephosphorylation of the phosphoenzyme is the rate-limiting step in both fast- and slow-twitch muscle (19). The activity as well as the concentration of the SR Ca^{2+}-pump exhibit a small but significant age-related decrease in human m. quadriceps (Fig. 1). The SR Ca^{2+}-ATPase content in adult muscle (6.7±1.1 nmol/g wet wt; $n = 29$) is similar to previously published data (19, 20). The SR Ca^{2+}-ATPase activity and content increase in cultured muscle cells with the maturation grade (Fig. 2), but remain lower than in muscle biopsies (Table II). However, the molecular activity, i.e., turnover number of ATP, of the SR Ca^{2+}-pump is equal in muscle and cultured cells (Table II).

The total SR Ca^{2+}-ATPase content was also measured in ELISA with a polyclonal antibody, raised against a mixture of both fast- and slow-type SR Ca^{2+}-ATPase. On base of the molecular mass (110 kD) the results (Table III) confirm the data obtained by phosphorylation (Table II). SERCA1, quantified by a specific monoclonal antibody, contributes 83 and 100% to the total SR Ca^{2+}-ATPase concentration in m. quadriceps
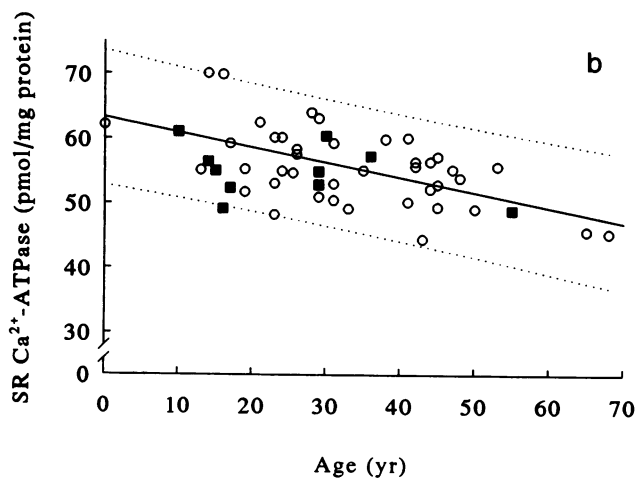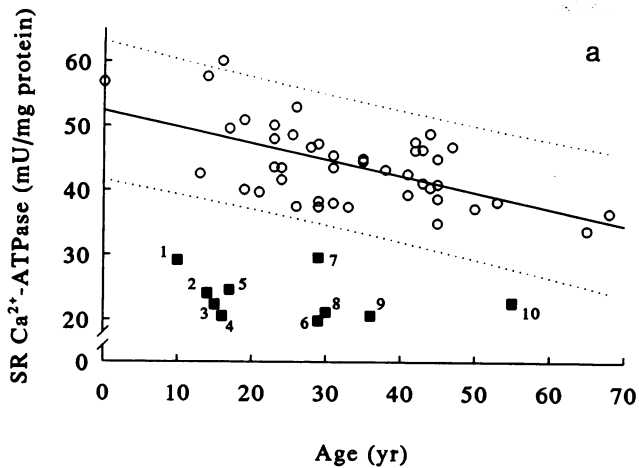
Figure 1. The effect of ageing on the SR Ca^{2+}-ATPase activity (a) and concentration (b) of control ($\bigcirc$) and pathological ($\blacksquare$) muscle. The solid lines represent the linear regression curves of the control values ($P <$ 0.01) and the dotted lines mark the 99% confidence intervals. The numbers correspond to the patient numbers as indicated in Table I.

Figure 2. The activity (a) and concentration of SR Ca^{2+}-ATPase (b) of cultured control ($\bigcirc$) and diseased ($\blacksquare$) muscle cells in relation to their maturation grade as expressed in percentage CK-MM. For other details see legend to Fig. 1.

and cultured muscle cells, respectively (Table III). For comparison we studied SR Ca^{2+}-ATPase activity and content in soleus muscle. The activity amounts to 20.2 ± 4.4 mU/mg protein and the total content and SERCA1 percentage are 3.30 ± 0.57 μg/mg protein and $50.3\pm10.1\%$, respectively ($n = 4$).

The activity of SR Ca^{2+}-ATPase is reduced by about 50% in muscle (Fig. 1 a) as well as cultured muscle cells (Fig. 2 a) of the patients compared to controls of the same age or maturation grade, respectively. However, the concentration of SR Ca^{2+}-ATPase is affected neither in muscle (Fig. 1 b) nor in cultured muscle cells (Fig. 2 b) of the patients. Besides, the concentration of the SERCA1 isoform is also normal in muscle as well as cultured cells of the patients. Consequently, the molecular activity of the SERCA1 isoform is $\sim$ 50% lower in the pathological muscle. The statistics are presented in Tables II and III. Immunocytochemical staining of SR Ca^{2+}-ATPase and SERCA1 did not reveal differences between muscle sections of controls and patients (data not shown). These results agree
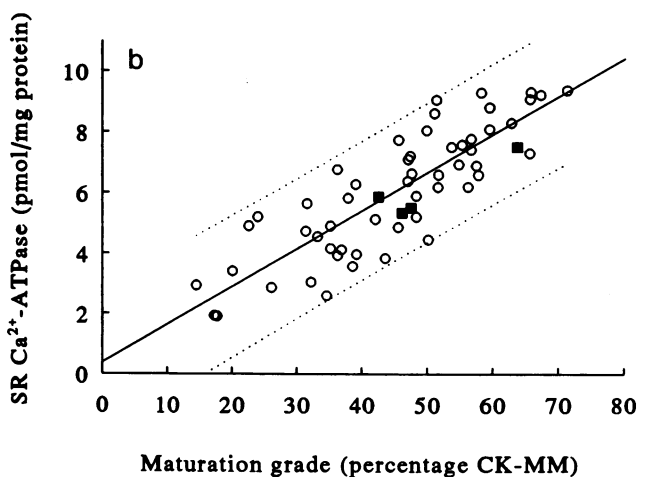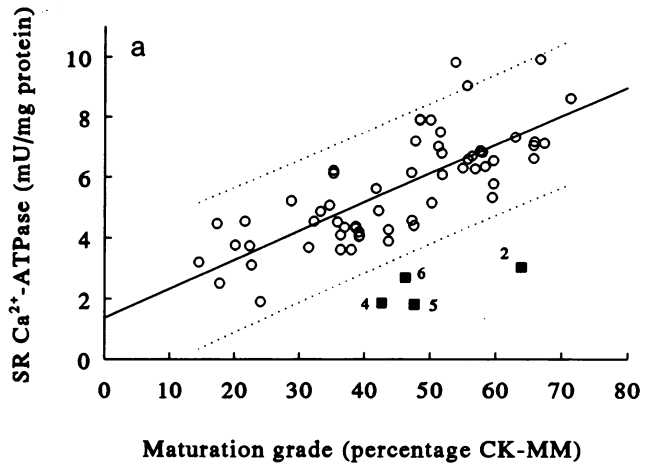
with the absence of changes in SR Ca^{2+}-ATPase content as quantified by phosphorylation and ELISA.

Na^+/K^+-ATPase. Na^+/K^+-ATPase activity is hardly detectable in homogenates of muscle biopsies, because of an excess of Mg^{2+}-ATPase activity. The K^+-dependent hydrolytic cleavage of the artificial substrate 3-O-methylfluorescein phosphate and its inhibition by ouabain can be used as a measure for Na^+/K^+-ATPase activity in muscle (21). Aging (14–58 yr) had no effect on the K^+-dependent 3-O-MFPase activity (data not shown). The K^+-dependent 3-O-MFPase activity raises in cultured muscle cells with their maturation grade (9) and is always higher than in muscle biopsies (Table II). In muscle and cultured muscle cells of the patients, the activity of K^+-dependent 3-O-MFPase is similar to controls of the same age or maturation grade, respectively (Table II).

Cytosolic Ca^{2+} concentration: the effects of acetylcholine, dantrolene, and verapamil. At the single cell level, $[Ca^{2+}]_i$ at rest in cultured muscle cells of controls is $\sim$ 130 nM (Fig. 3, a and e). After addition of 300 μM acetylcholine (ACh) in the presence or absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ rises to 400

Table II. SR Ca²⁺-ATPase and K⁺-dependent 3-O-MFPase in Muscle and Cultured Muscle Cells of Controls and Patients Suffering from Brody's Disease

Parameters	Muscle		Muscle cultures	
	Controls	Patients	Controls	Patients
SR Ca²⁺-ATPase$_{(activity)}$ (mU/mg protein)	43.9 ± 6.0 (44)	23.4 ± 3.5* (10)	6.46 ± 1.40 (28)	2.33 ± 0.61* (4)
SR Ca²⁺-ATPase$_{(phosphorylation)}$ (pmol/mg protein)	55.5 ± 5.9 (41)	54.7 ± 4.2 (10)	6.93 ± 1.39 (28)	6.04 ± 0.99 (4)
Molecular activity (min⁻¹)	810 ± 87 (41)	411 ± 49* (10)	892 ± 127 (25)	397 ± 98* (4)
K⁺-dependent 3-O-MFPase (mU/mg protein)	0.39 ± 0.10 (28)	0.38 ± 0.06 (10)	1.08 ± 0.19 (25)	1.05 ± 0.12 (4)

Values are means±SD of the number of individuals given between parentheses. Units of activity represent μmol ATP or 3-O-MFP hydrolysed per min at 37°C. The maturation grade (percentage CK-MM) of cultured muscle cells of controls and patients varied between 42.2 and 63.8%. Parameters are significantly different from controls with: $*P < 0.01$.

nM (Fig. 3, *b* and *f*). The maximal response is reduced to ~ 50% within 20 s (Fig. 3, *c* and *g*) and the resting [Ca²⁺]$_i$ is restored 1 min after stimulation (Fig. 3, *d* and *h*). In cultured muscle cells of patients the basal [Ca²⁺]$_i$ is comparable with the value of controls (Fig. 3, *i* and *m*). The maximal [Ca²⁺]$_i$ is similar too after ACh stimulation (Fig. 3, *j* and *n*) and also independent of extracellular Ca²⁺ (data not shown). However, 20 s after the maximal response, [Ca²⁺]$_i$ is still about 340 nM (Fig. 3, *k* and *o*) and after 1 min the basal value is not yet reached (Fig. 3, *l* and *p*). The cell-to-cell variability in these [Ca²⁺]$_i$ measurements varies from 3 to 9% in cells of controls as well as patients.

To resolve the response times, experiments were performed using the NPS Photon Counting system. Fig. 4 shows the temporal differences in [Ca²⁺]$_i$ upon stimulation in control and pathological cells. For both cell types the increase and decay phase of the Ca²⁺ transients can be fitted by the mono-exponential Eqs. 1 and 2, respectively:

$$[Ca^{2+}]_i = [Ca^{2+}]_{i,basal} + A \cdot \{1 - e^{(-t/\tau_i)}\} \quad (1)$$

$$[Ca^{2+}]_i = [Ca^{2+}]_{i,plateau} + B \cdot e^{(-t/\tau_d)} \quad (2)$$

using SlideWrite Plus 5.00 (Advanced Graphics Software, Carlsbad, CA) as illustrated in Fig. 4 *a*. The constant A equals {1 − e$^{((t_a-t)/\tau_i)}$} · {[Ca²⁺]$_{i,max}$ − [Ca²⁺]$_{i,basal}$} and B equals

e$^{(t_{max}/\tau_d)}$ · {[Ca²⁺]$_{i,max}$ − [Ca²⁺]$_{i,plateau}$}, in which t$_a$ and t$_{max}$ are the times at which ACh is added or the maximal Ca²⁺ concentration, i.e., [Ca²⁺]$_{i,max}$, is reached, respectively. The half-life of the increase phase (τ_i) in muscle cells of controls and patients are 5 to 6 s, whereas the halflife of the decay phase (τ_d) are ~ 20 and 60 s, respectively. In pathological cells it takes more than 5 min before the basal [Ca²⁺]$_i$ is reinstalled. The addition of 10 μM dantrolene or verapamil at 4 min after the maximal response accelerates the restoration of the basal [Ca²⁺]$_i$ in these cells within 23 to 41 s instead of 2 to 3 min in the absence of the drugs (Fig. 4, *b* and *c*). At simultaneous supply of ACh and dantrolene or verapamil the maximal [Ca²⁺]$_i$ (240 nM), τ_i (3 s) as well as τ_d (30 s) are equal in muscle cells of controls and patients (Fig. 5). Table IV gives a statistical evaluation of the data.

Discussion

Brody's disease is a rare inherited error of muscle function. Clinical diagnosis is quite difficult, since the signs and symptoms of the patients are rather heterogeneous and nonspecific. Even impaired muscle relaxation can be absent in some cases as in our patients 1, 2, 5, and 9 and in a patient described by Taylor et al. (4). Therefore the diagnosis, Brody's disease,

Table III. SR Ca²⁺-ATPase and SERCA1 Content in Muscle and Cultured Muscle Cells of Controls and Patients Suffering from Brody's Disease

Parameters	Muscle		Muscle cultures	
	Controls	Patients	Controls	Patients
SR Ca²⁺-ATPase (μg/mg protein)	6.12 ± 0.75 (22)	6.11 ± 0.41 (10)	0.43 ± 0.09 (14)	0.43 ± 0.12 (4)
SERCA1 (μg/mg protein)	5.06 ± 0.57 (22)	5.09 ± 0.36 (10)	0.42 ± 0.08 (14)	0.43 ± 0.09 (4)
SERCA1 (%)	82.8 ± 4.6 (22)	83.4 ± 3.9 (10)	99.3 ± 7.9 (14)	102 ± 8.5 (4)

Values are means±SD of the number of individuals given between parentheses. Total SR Ca²⁺-ATPase was determined with a polyclonal antibody against both SERCA isoforms, SERCA1 with a monoclonal antibody (5D2) specific for this isoform. For other details see legend to Table II.
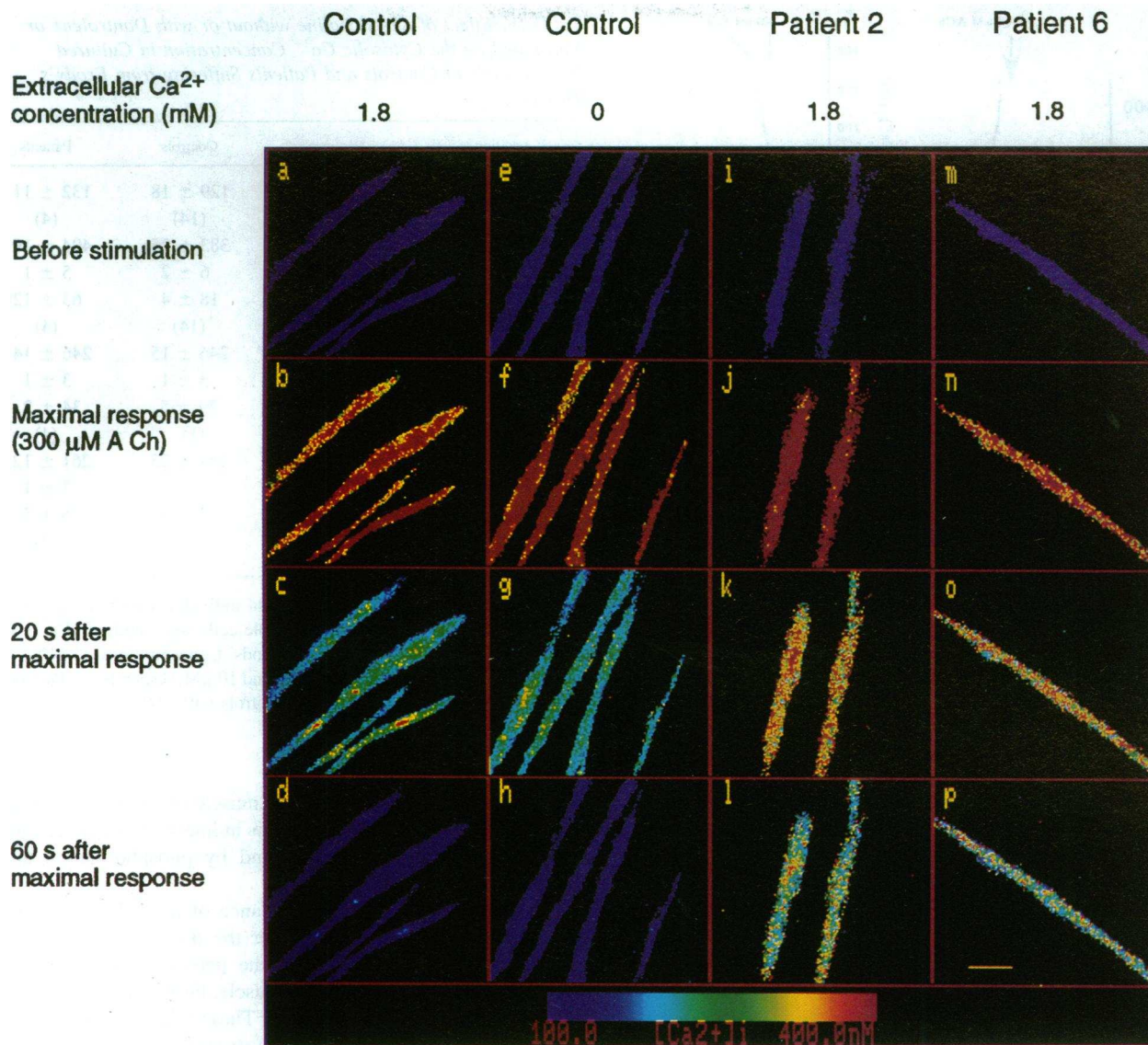
Figure 3. Temporal and spatial distribution of cytosolic Ca^{2+} in cultured muscle cells of controls ($a-h$), patient 2 ($i-l$) and patient 6 ($m-p$) before (a, e, i, and m) and after stimulation with ACh. All experiments are performed in the presence of extracellular Ca^{2+} (1.8 mM), except $e-h$ which are done under Ca^{2+}-free conditions (PSS containing 0.5 mM EGTA and no Ca^{2+}). For other details see text.

should be considered more commonly in patients with exercise-induced muscle stiffness, cramp and pain syndromes.

Quantitative data on SR Ca^{2+}-ATPase are difficult to compare, because various authors used a wide range of techniques. In only 2 of the 12 patients presented in literature the activity as well as concentration of the SR Ca^{2+}-pump were biochemically characterized (2, 4). To gain an insight into the pathogenesis of Brody's disease it is necessary to examine both the activity and concentration of the SR Ca^{2+}-pump. SR Ca^{2+}-ATPase activity or ATP-dependent Ca^{2+} uptake were determined with isolated microsomal or SR fractions and varied between 2 and 30% of the control values (1, 2, 4). The preparation of microsomal or SR fractions may introduce, however, differences in recovery of the SR Ca^{2+}-pump. Assay of SR Ca^{2+}-ATPase activity and protein content can therefore better be performed on whole muscle homogenates. The residual SR Ca^{2+}-ATPase activity in whole muscle homogenates of patients is ~ 50% (5, 6) (Table II).

SR Ca^{2+}-ATPase constitutes ~ 90% of the total protein content in SR membrane (22). Its content in human skeletal muscle depends on muscle type and age. The concentration of SR Ca^{2+}-ATPase is twice as high in m. quadriceps as in m. soleus. On base of the fiber type composition of both muscles (60 and 20% fast-twitch fibers, respectively) it can be deduced that the SR Ca^{2+}-ATPase content is about six times higher in fast-twitch than in slow-twitch fibers. This is in line with cytochemical data (9). The decrease of the activity and concentration of SR Ca^{2+}-ATPase in human skeletal muscle upon ageing is associated with a selective atrophy of fast-twitch fibers (23, 24). In both muscle and cultured muscle cells of our patients the decrease of SR Ca^{2+}-ATPase activity is not caused by a reduction of the SR Ca^{2+}-ATPase protein content, which agrees with the results of Taylor et al. (4). Moreover, the contribution of SERCA1 to the total SR Ca^{2+}-ATPase content is comparable in controls and patients, so the possibility that the SR Ca^{2+}-ATPase deficiency is related to an isoform switch of SERCA1 to SERCA2 can be excluded. This implies that the molecular activity of the SERCA1 isoform is reduced.
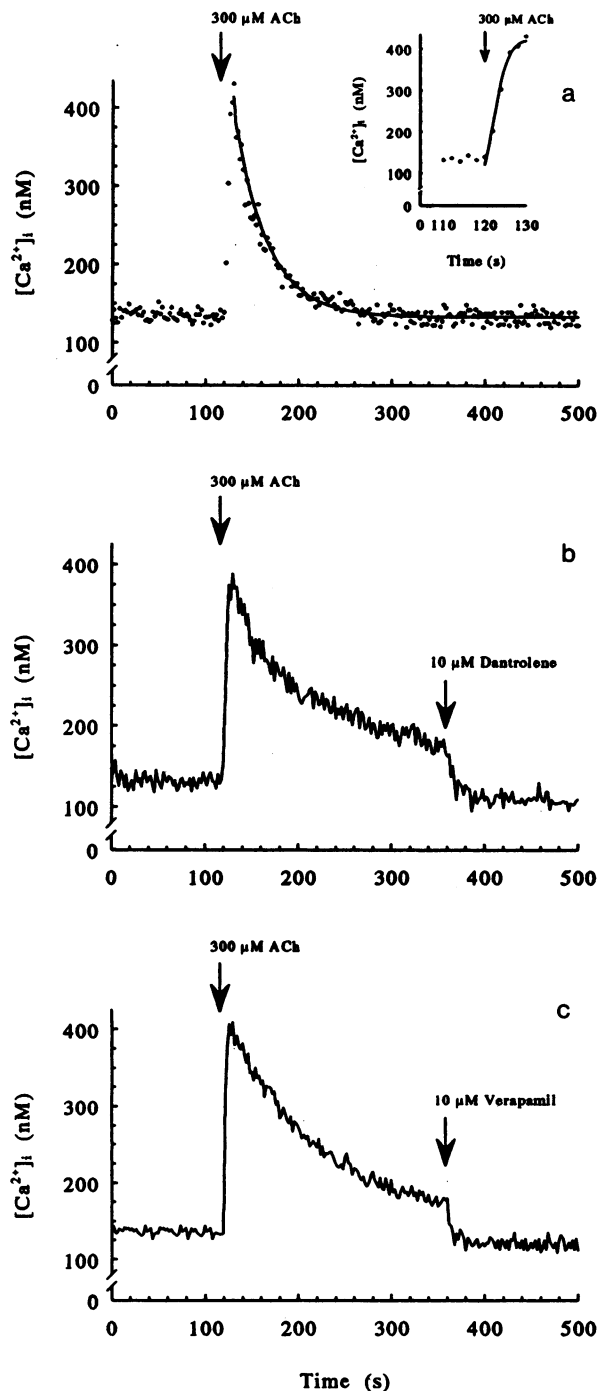
Figure 4. The Ca^{2+} response in control (a) and diseased muscle cells (b and c) upon the addition of ACh and the effect of dantrolene and verapamil. (a) The solid lines represent the mono-exponential curves, which fit the increase (*inset*) and decay phase of the Ca^{2+} transient. For other details see text.

Other investigators detected by immuno(cyto)chemical techniques a marked decrease of the SERCA1 content (2, 3). These analyses were based on poly- or monoclonal antibodies against chicken SR Ca^{2+}-ATPase, which might be incompetent to react with the SR Ca^{2+}-pump of the pathological muscle, due to structural changes in the protein which may underlay the altered molecular activity. However, we did not observe a

Table IV. Effect of Acetylcholine without or with Dantrolene or Verapamil on the Cytosolic Ca^{2+} Concentration in Cultured Muscle Cells of Controls and Patients Suffering from Brody's Disease

Addition	Parameters	Controls	Patients
None	$[Ca^{2+}]_i$	129 ± 18	132 ± 11
		(14)	(4)
ACh	$[Ca^{2+}]_{i,max}$	383 ± 25	404 ± 19
	τ_i	6 ± 2	5 ± 1
	τ_d	18 ± 4	63 ± 12*
		(14)	(4)
ACh + dantrolene	$[Ca^{2+}]_{i,max}$	246 ± 15	246 ± 14
	τ_i	3 ± 1	3 ± 1
	τ_d	29 ± 5	34 ± 2
		(8)	(4)
ACh + verapamil	$[Ca^{2+}]_{i,max}$	257 ± 23	261 ± 12
	τ_i	3 ± 1	3 ± 1
	τ_d	32 ± 4	35 ± 3
		(4)	(3)

Values are means±SD of the number of individuals given between parentheses. For each culture 5–7 whole cells were studied. $[Ca^{2+}]_i$ is expressed in nM and τ_i and τ_d in seconds. Concentrations of ACh, dantrolene or verapamil were 300, 10, and 10 μM, respectively. Parameters are significantly different from controls with: *$P < 0.01$.

decrease in staining of any type of muscle fibers with our poly- and monoclonal antibodies, which is in line with the unchanged SR Ca^{2+}-ATPase content as found by phosphorylation and ELISA.

In Brody's disease the disturbance of the SR Ca^{2+}-pump seems to be specific, since neither the fiber-type distribution nor the total protein content of the pathological muscle are modified compared with control muscle. Furthermore, the Na^+/ K^+-ATPase activity is normal too. These results are in contrast to our observations in myotonic dystrophy (28). Both the SR Ca^{2+}-ATPase and the Na^+/K^+-ATPase activity are decreased in muscle and cultured muscle cells of myotonic dystrophy patients, due to a reduced number of these ion pumps.

Identification of the molecular defect(s) in Brody's disease is subject of our current research. Substitutions of certain amino acids by site-directed mutagenesis in the stalk, hinge or trans-membrane domains of SR Ca^{2+}-ATPase induce a marked reduction of Ca^{2+} transport and ATPase activity without affecting phosphorylation (25–27). Other mutations inhibit phosphorylation of the SR Ca^{2+}-pump. It remains to be elucidated whether and in which way the SERCA1 gene on chromosome 16 (29) is involved in Brody's disease.

Since the deficiency is expressed in the same way in muscle and cultured muscle cells it was of interest to examine the Ca^{2+} homeostasis in these cells to understand the consequences of SR Ca^{2+}-ATPase deficiency for cell functioning. In addition, this in vitro model could be used to investigate the effects of potential therapeutics. We were unable to study Ca^{2+} homeostasis in separate fibers 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 (30). $[Ca^{2+}]_i$ at rest and after ACh stimulation in cultured muscle cells of controls are comparable with published data (13, 31,
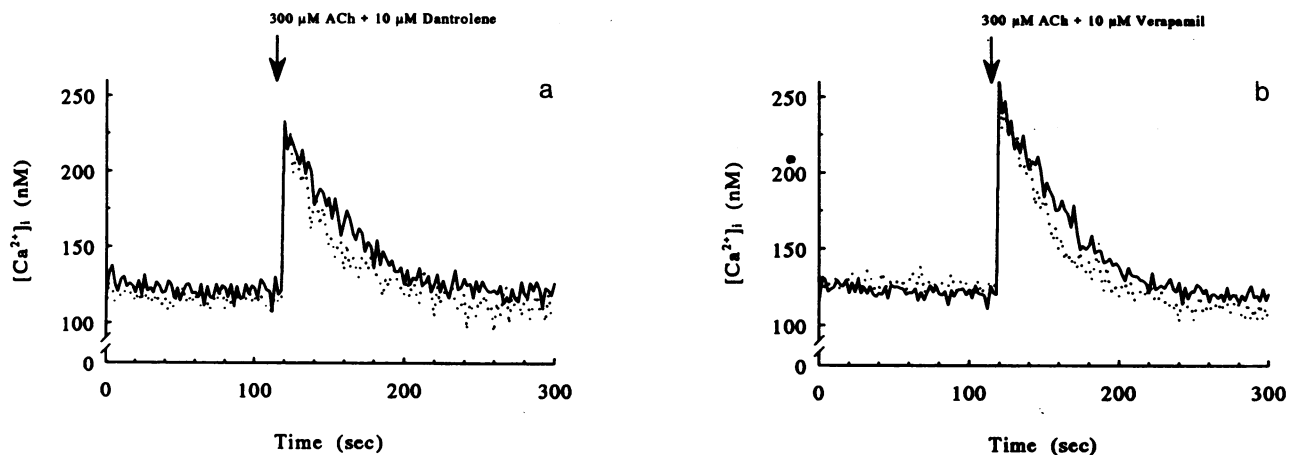
300 μM ACh + 10 μM Dantrolene

300 μM ACh + 10 μM Verapamil

Figure 5. The effect of ACh on the $[Ca^{2+}]_i$ of control (*dotted traces*) and pathological muscle cells (*solid traces*) in the presence of dantrolene (*a*) or verapamil (*b*).

32). Since maximal $[Ca^{2+}]_i$ after stimulation with ACh is equal in the presence or absence of extracellular Ca^{2+}, the rise of cytosolic Ca^{2+} originates from Ca^{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, in which a Ca^{2+}-induced Ca^{2+} release mechanism causes the signal transduction (33).

In the cultured muscle cells of the patients maximal $[Ca^{2+}]_i$ after ACh stimulation is similar to that of control cells. Besides, the halflifes of $[Ca^{2+}]_i$ increase are the same for both types of cells. Thus, the depolarization of the muscle cell membrane induced by ACh and the subsequent Ca^{2+} release from the SR are normal in the pathological muscle cells, which is in agreement with the in vivo situation. In electromyography, patients show a physiological recruitment of normal motor unit potentials (1, 2, 4, 5). The shape of the Ca^{2+} transient is defined by two opposite acting mechanisms. On one hand, in the presence of ACh the plasma membrane is depolarized and the dihydropyridine receptor–ryanodine receptor complex mediates the Ca^{2+} release from the SR. On the other hand, the SR Ca^{2+}-pump, which is activated by the increased cytosolic Ca^{2+} concentration, restores the $[Ca^{2+}]_i$. In the muscle cells of the patients the increased time needed for Ca^{2+} extrusion from the cytosol after excitation is a consequence of the decreased activity of the SR Ca^{2+}-pump, resulting in pseudo-myotonia in vivo. At addition of dantrolene or verapamil, blockers of the dihydropyridine receptor–ryanodine receptor complex (34, 35), the release of Ca^{2+} is inhibited, but the SR Ca^{2+}-pump is still active and the restoration of the basal $[Ca^{2+}]_i$ becomes accelerated. It is not very likely that at this point ($[Ca^{2+}]_i$ is $\sim$ 200 nM) other Ca^{2+}-restoring processes, e.g., the sarcolemmal Ca^{2+}-ATPase and the Na^+/Ca^{2+}-exchanger may be involved in the reinstallation of the $[Ca^{2+}]_i$.

The sarcolemmal Ca^{2+}-pump amounts to only 10% of the total Ca^{2+}-dependent ATPase activity in control cells (9, 28) and probably $\sim$ 20% in the muscle cells of the patients as a result of the by 50% decreased SR Ca^{2+}-ATPase activity. Moreover, the sarcolemmal Ca^{2+}-ATPase has a lower affinity for $[Ca^{2+}]_i$ than the SR Ca^{2+}-pump (36, 37). Relatively little is known about the Na^+/Ca^{2+}-exchanger in skeletal muscle. This antiporter uses the energy from the Na^+ electrochemical potential gradient to extrude Ca^{2+} from the cell against a large Ca^{2+} gradient (36). Accepting a $[Ca^{2+}]_i$ of 200 nM, a cytosolic

Na^+ concentration of 12.4 mM (unpublished results), extracellular concentrations of Ca^{2+} and Na^+ of 1.8 and 136 mM, respectively, and a membrane potential of −60 mV (38) the electrochemical potential gradient of Ca^{2+} and Na^+ can be calculated (39) to be 35 kJ/mol and 12 kJ/mol, respectively. Assuming a stoichiometry of 3 Na^+ versus 1 Ca^{2+} (36), the two electrochemical potential gradients are balanced, which implies that the Na^+/Ca^{2+}-exchanger will not be functioning.

At simultaneous supply of ACh and dantrolene or verapamil the Ca^{2+} response is reduced. Due to the lower maximal $[Ca^{2+}]_i$, SR Ca^{2+}-ATPase is less activated (9) and the halflife of the Ca^{2+} decay is increased in control cells. However, under these circumstances less Ca^{2+} pumping capacity is required to restore the $[Ca^{2+}]_i$ and the normalization of the $[Ca^{2+}]_i$ after excitation is comparable in cultured muscle cells of controls and patients. So if a Ca^{2+} overload is prevented in the pathological cells, they apparently are able to handle the Ca^{2+} response as control cells do. These observations may explain the beneficial effect of administration of dantrolene (5, 6) or verapamil (4) to patients suffering from Brody's disease.

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

Acknowledgments

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