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Original Article

Contractile elements in muscular fascial tissue – implications for in-vitro contracture testing for malignant hyperthermia

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Summary

Malignant hyperthermia is a dreaded complication of general anaesthesia. Predisposed individuals can be identified using the standardised caffeine/halothane in-vitro contracture test on a surgically dissected skeletal muscle specimen. Skeletal muscle is composed of muscle fibres and interwoven fascial components. Several malignant hyperthermia-associated neuromuscular diseases are associated with an altered connective tissue composition. We analysed adjacent fascial components of skeletal muscle histologically and physiologically. We investigated whether the fascial tissue is sensitive to electrical or pharmacological stimulation in a way similar to the in-vitro contracture test for diagnosing malignant hyperthermia. Using immunohistochemical staining, α-smooth muscle actin-positive cells (myofibroblasts) were detected in the epi-, endo- and perimysium of human fascial tissue. Force measurements on isolated fascial strips after pharmacological challenge with mepyramin revealed that myofascial tissue is actively regulated by myofibroblasts, thereby influencing the biomechanical properties of skeletal muscle. Absence of electrical reactivity and insensitivity to caffeine and halothane suggests that, reassuringly, the malignant hyperthermia diagnostic in-vitro contracture test is not influenced by the muscular fascial tissue.

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Introduction

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder, which presents as hypermetabolism, severe acidosis, tachycardia, rapidly increasing body temperature and rhabdomyolysis [1]. The syndrome is triggered by halogenated anaesthetics and the depolarising neuromuscular blocking drug suxamethonium [2]. The pathological mechanism of MH is an abnormally increased release of calcium from the sarcoplasmic reticulum by a disrupted ryanodine receptor type-1 function [2, 3]. The excessive calcium release can be detected as abnormal contracture in surgically dissected muscle bundles in the so-called in-vitro contracture test.

The protocols of the North American (NAMHG) and European MH group (EMHG) include exact information on preparation of a muscle specimen, performance of measurements and interpretation of results [3–5]. The muscle biopsy is optimally taken near the surface of vastus muscle, specifically the centre portion, due to parallel orientation of bundles. Here, the

specimens commonly contain fascial tissue (endo-, peri- and epimysium). This connective tissue is considered to serve a passive role in biomechanical dynamics, transmitting mechanical tension. However, recent data from animal studies suggest that fascial tissue might have contractile properties [6, 7].

Several neuromuscular diseases are associated with increasing amounts of connective tissue, and an above average coincidence of clinical MH-suspicion and disorders of connective tissue as well as musculoskeletal disorders has been suggested [8, 9]. Nonetheless, the current protocols do not address the content and properties of fascial tissue in the biopsy specimens. Hence, it is important to exclude the possibility that fascial components of skeletal muscle are sensitive to electrical or pharmacological stimulation, or that the mechanical properties of connective tissue might influence the results of the MH-diagnostic halothane-caffeine contracture test.

Methods

Biopsies of human musculus vastus lateralis were dissected under regional anaesthesia for in-vitro contracture test according to the guidelines of the EMHG and with the approval of the local ethics committee of Ulm University. The indications were MH-crisis of the patient, family trait of MH and chronic isolated creatine kinase elevations. Tissue samples of lumbar fascia of 19 mice (type BALB/cJ) and eight rats (three Hooded Lister, five Wistar) were also used according to the local ethics committee of Ulm University. For mechanographic force recording, the tissue was used fresh and for immunohistochemical processing, all tissue specimens were fixed in 5% neutral buffered formalin and embedded in paraffin.

For immunohistochemistry, paraffin specimens were cut into 2.5-μm thick sections and mounted on glass slides coated with 3% silane. The slides were then de-paraffinised with xylol and immersed in methanol at decreasing concentrations (100%, 96%, 70%) containing 0.3% hydrogen peroxide for a total of 4 min. After pre-treatment with 0.1% trypsin (Sigma-Aldrich, Steinheim, Germany) for 30 min, sections were blocked with 20% goat serum (Sigma-Aldrich). Finally, sections were incubated with mouse monoclonal primary antibody to α-smooth muscle actin (catalogue no. AM128; BioGenex, San Ramon, CA, USA) at 4 °C for 1 h.

For immunofluorescence, paraffined sections were cut at 10 μm and mounted on glass microscopic slides, which were then de-paraffinised with xylol and immersed in ethanol at decreasing concentrations (100%, 96%, 80%, 70%, 50% each for 1 min) followed by immersion in distilled water for 3 min and then washed 3 × 10 min with phosphate-buffered saline. After incubating with mouse monoclonal primary antibody to α-smooth muscle actin 1 (mouse IgG2a mAB, University Geneva, Gabbiani G, Switzerland) for 1 h, sections were washed 3 × 10 min with phosphate-buffered saline and subsequently probed with goat anti-mouse-Alexa 488 (catalogue no. A-11029; Molecular Probes, Eugene, OR, USA) in phosphate-buffered saline at room temperature in darkness (50 μl per coverslip). Cell nuclei were stained with DNA probe DAPI (catalogue no. 32670; Sigma-Aldrich). Finally, sections were washed 3 × 30 min with phosphate-buffered saline plus 1 min with distilled water both at room temperature in darkness and overlaid with one drop of polyvinylalcohol as a mounting liquid.

Slides were analysed with a Zeiss Axiophot microscope using polychromatic and polarised light and equipped with Plan-Neofluoar ×20/0.50 objective (Carl Zeiss, Eching, Germany). The microscope was connected to a digital image analysis system (Diskus Version 4.5; C.H. Hilgers, Königswinter, Germany), linked to a digital colour camera (JVC KY-F75U; Victor Company of Japan, Yokohama, Japan) with an output signal resolution of 1360 × 1024 pixels. Except for some slides from non-human tissue, all slides were randomly coded for blinded investigation. Vascular smooth muscle cells on the same slide were used as the positive control, matching the immunohistochemical labelling of α-smooth muscle actin containing cells or fibres. Pictures were then processed digitally, using software specifically developed for this study (written in Delphi 3.0; Borland International, Scotts Valley, CA, USA). This software counted the number of brown pixels and also reported them as a fraction from the total number of pixels in the image (width × height).

Human biopsies of vastus lateralis muscle or fresh fascial tissues from mice (BALB/cJ; 25–35 g) and rats (Wistar; 250–360 g/ Hooded Lister; 270–450 g) were mounted in an organ bath and continuously bubbled

with carbogen (95% O_2, 5% CO_2, MTI IndustrieGASE, Neu-Ulm, Germany). Mechanographic experiments were conducted as described previously [10]. For experiments with isolated animal fascial tissue, all visible muscle fibres were removed from the fascial tissue with a surgical knife and controlled via light microscope with 20× magnification. The solution in the bath was kept at a constant temperature of 35 °C for human samples and 25 °C for murine samples. Signal recording was performed using special software (written in Delphi 1.0; Borland International) [5]. A pair of platinum electrodes was placed on the lateral parts of the fascia for electrical stimulation with supramaximal stimuli (70 V, pulse-width 1 ms, 0.2, 5 and 20 Hz). Muscle bundles were pre-stretched using optimum force development to roughly 150% of their initial length. Mechanographic measurements of fascia were conducted with a 4% strain. All experiments started after allowing the specimens to equilibrate in the chamber solution for a period of at least 15 min. Control tissues, made unviable with repetitive deep freezing and rapid thawing, were used for comparison [11, 12]. Pharmacological effects of the nitric oxide donor substance glyceryl trinitrate and mepyramine as known substances with dilatory or contractile effects on smooth muscle were tested on fascial tissue and added to the organ bath in concentrations of 0.1–1 mM glyceryl trinitrate and 1–25 mM mepyramine. A stock solution (100 mM) of caffeine was prepared afresh and added stepwise to the tissue bath to yield concentrations in the range of 0.5–32 mM. Halothane was applied to the tissue baths using a vaporiser (Vapor 19.1 Draeger, Luebeck, Germany) with concentrations of 0.5% to 4% v/v. Contracture curves were displayed and recorded with a computer-based data evaluation program. For further details, see Ording et al. [6].

In the quantitative analysis of histological data, a Mann–Whitney test (U-test) was used to test significant differences between samples from different individuals. A Wilcoxon test was used to test for significant differences between different sample locations of the same group of individuals. A level of $p < 0.05$ was accepted as statistically significant.

Results

The human muscle samples (n = 11) contained different amounts of connective tissue within and surrounding the specimens, with macroscopically apparent differences in the amount of adherent and interwoven connective tissue (Fig. 1). Histologically, Fig. 2 shows in a sample cross-section of a myofascial unit that the perimysium and, to a lesser extent, epi- and endomysium, contained α-smooth muscle actin-positive cells, namely myofibroblasts.

In electrical stimulation (n = 25) of mice specimens, a frequency of 0.2 Hz (as used for in-vitro contracture testing) resulted in no force change. At all tested frequencies (0.2, 5, 20 Hz), there was no significant force reaction or contracture development (Fig. 4a). Counter-intuitively, a slight increase in force at 5 Hz and a slight reduction at 20 Hz were observed; however, this was attributed to fluid perturbations related to the frequency (confirmed by using control experiments using unviable tissue; results not shown).

Pharmacological challenge of fascial tissue with nitric oxide donor glyceryl trinitrate exhibited a relaxing effect on fascial tissue (Fig. 3a). A concentration of 1×10^{-4} M in the organ bath resulted in a force reduction in dorsal fascia from mice, median (IQR [range]) −1.0 (−0.8 to −1 [0 to −4.5]) mN. A higher concentration of 5×10^{-4} M led to a stronger response: −2.0 (−1 to −2.9 [0 to −6]) mN. A similar relaxation response was observed in human fascia of vastus lateralis muscle after addition of 5×10^{-4} M: −3.6 (−3.5 to −5.7 [−2.9 to −7.2]) mN.

The addition of mepyramine to the organ bath yielded a clear contractile effect on fascial tissue. Rat lumbar fascia responded to 10×10^{-3} M in 13 of 24 tests with a force increase: 3.6 mN (7.0−1.5 [13.5−0.39]) mN. Figure 3b shows the typical slow and long-lasting response curve observed in most of the tests. Mean (SD) time between substance addition and maximum contraction was 63 (35) min. From two samples of human vastus lateralis fascia, one sample contracted in response to 250×10^{-3} M with 1.8 mN. The long duration of the response curves was surprising. For comparison, 13 tests were performed with mepyramine application to pieces of unviable control fascia (rat lumbar fascia, pre-treated using freeze-thaw cycles). As expected, there was no force response for these tissues.

In eight samples of rat lumbar fascia and 10 samples of fascia from human vastus lateralis, there was

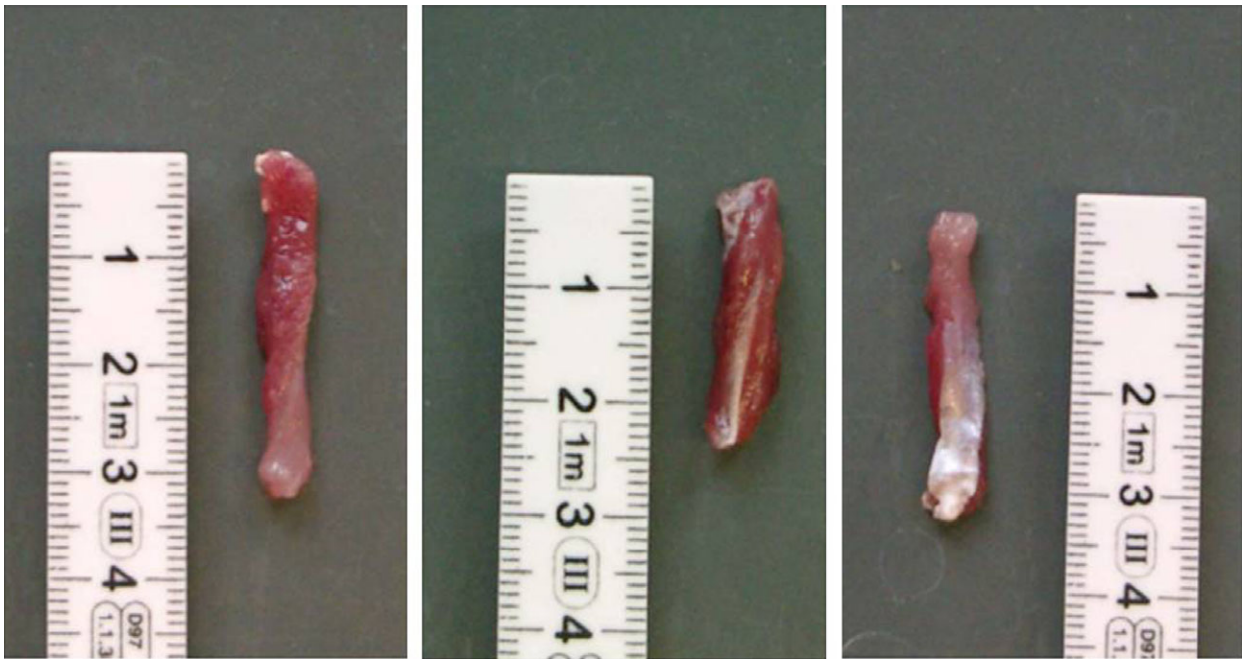

Figure 1 Three typical muscle biopsies of the human vastus lateralis as usually taken for in-vitro contracture investigation, showing different compositions (exhibited by different proportions of red/white colouring) of skeletal muscle fibres and fascial connective tissue.

no significant force alteration in response to caffeine and halothane. Most notably, contractures could not be observed in the vastus lateralis fascia of MH-susceptible individuals (n = 6).

A concentration of 32 mM caffeine is used in the in-vitro contracture test to confirm the viability of muscle tissue. If this control concentration does not cause the expected contraction, the in-vitro contracture test is interpreted as an invalid test result, as muscle samples are not able to contract. Exposing fascial tissue to the control concentration of caffeine, we detected slight but insignificant changes of tension (0 (0 to −1 [0 to −4]) mN (Fig. 4b). Caffeine is a weak xanthine base and altered the pH of the used physiological KR solution at a concentration of 32 mM by −0.1 to –0.2, resulting in pH values of −7.5 to –7.6 within the organ bath. Moreover, we found neither functional nor immunohistochemical differences between non-MH-susceptible patients and MH-susceptible.

Discussion

We confirmed that there is an active smooth muscle-like contractility of fascial tissue, which might potentially influence the results of MH testing. An unpublished survey of various malignant hyperthermia test centres has revealed very different handling procedures for fascial connective tissue (personal observations). Although removal of connective tissue via dissection might permit more exact measurements of muscle force, the removal of all fascial tissues is not always performed and moreover might harm muscle fibres. However, insensitivity to electrical stimulation and to the pharmacological stimulation with caffeine and halothane, as observed in our experiments, suggests – reassuringly – that the MH-diagnostic in-vitro contracture test is not influenced by the fascial tissue.

Our results support the notion that contractions of myofibroblasts are independent of ryanodin receptor stimulation. In MH muscle, excessive release of calcium ions (Ca^{2+}) through an abnormal RyR1 protein in the presence of the potent RyR1 agonists halothane or caffeine stimulates the contractile apparatus and results in skeletal muscle contracture [3, 13, 14]. Caffeine mediates its pharmacological effects in increasing doses as an antagonist of the adenosine receptor, via the activation of ryanodine receptor type-1 mechanism and direct activation of the actin-myosin filaments [15, 16]. However, caffeine induced

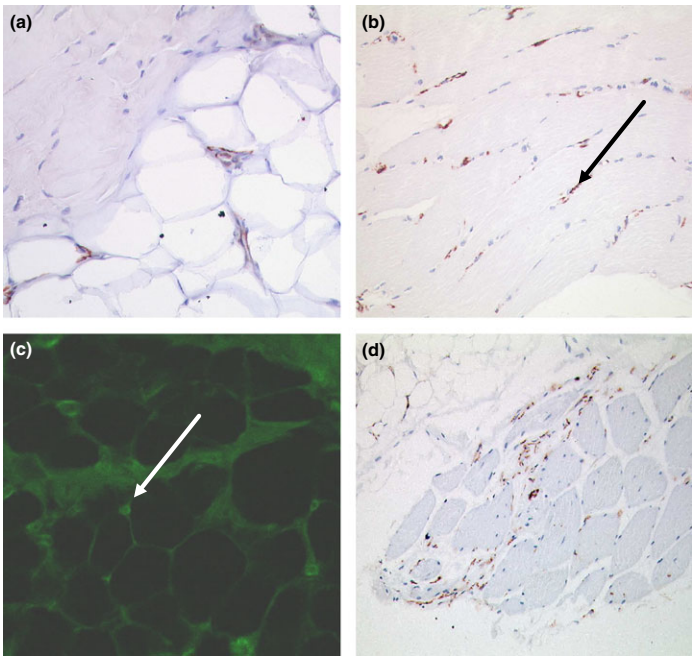

Figure 2 Histological slides of rat fascia. (a) Example of alpha smooth muscle actin-stained fibres near fat cells. Image length 250 μM. (b) Example of increased density of alpha smooth muscle actin-stained fibres in intramuscular fascia (e.g. marked with an arrow). Image length 400 μM. (c) Immunofluorescence imaging of intramuscular fascia. Bright green: alpha smooth muscle actin labelling. Note the clearly stained line from the upper right towards the upper left side, indicating a high density of contractile cells in this perimysium. Example of a typical myofibroblast is marked with an arrow. Image length 550 μM. (d) Example of perimysial fascia. The fat cell area on the upper left side also contains some alpha smooth muscle actin-stained elements. Image length 400 μM.

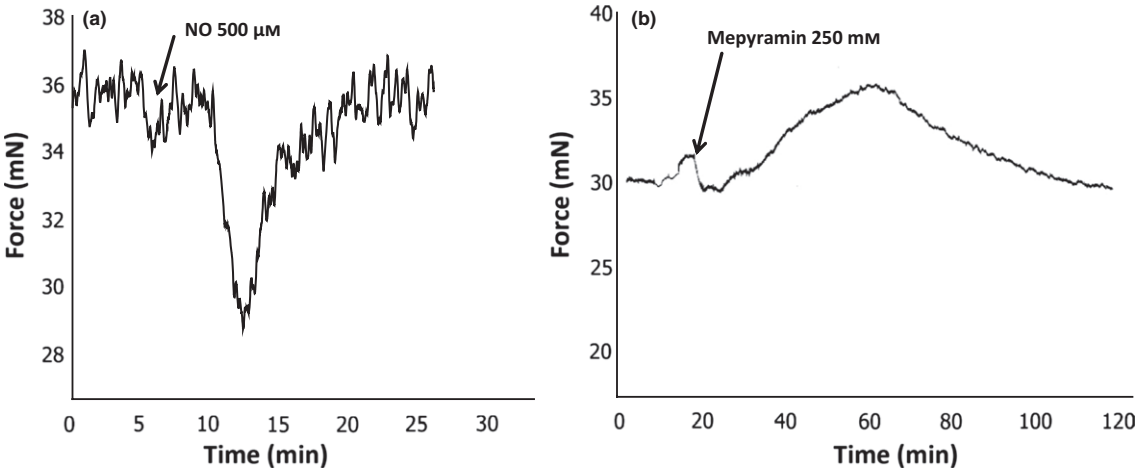

Figure 3 Responses of fascial tissue to pharmacological agents. (a) Relaxation effect of the nitric oxide donor glyceryl trinitrate on human fascia lata. The nitric oxide donor is added to the immersion bath at 5 min. Resulting substance concentration in the bath is 5×10^{-4}M. Sample size 2.5 mm^2 × 20 mm. A clear decrease in force is observed 5 min after substance addition, lasting approx. 10 min before recovering to the baseline. (b) Contractile effect of mepyramine. A bundle of rat lumbar fascia is exposed to 250 mM mepyramine in superfusion. The brief initial force increase is due to temporary gain of the tissue due to the more sticky mepyramine solution, which is then quickly washed off. A typical feature of the response of fascial tissue to mepyramine is the slow and sustained duration of the reaction. Bundle size 31mm × 12 mm^2. (In control experiments with tissue that was frozen and thawed before testing, we found no contraction).

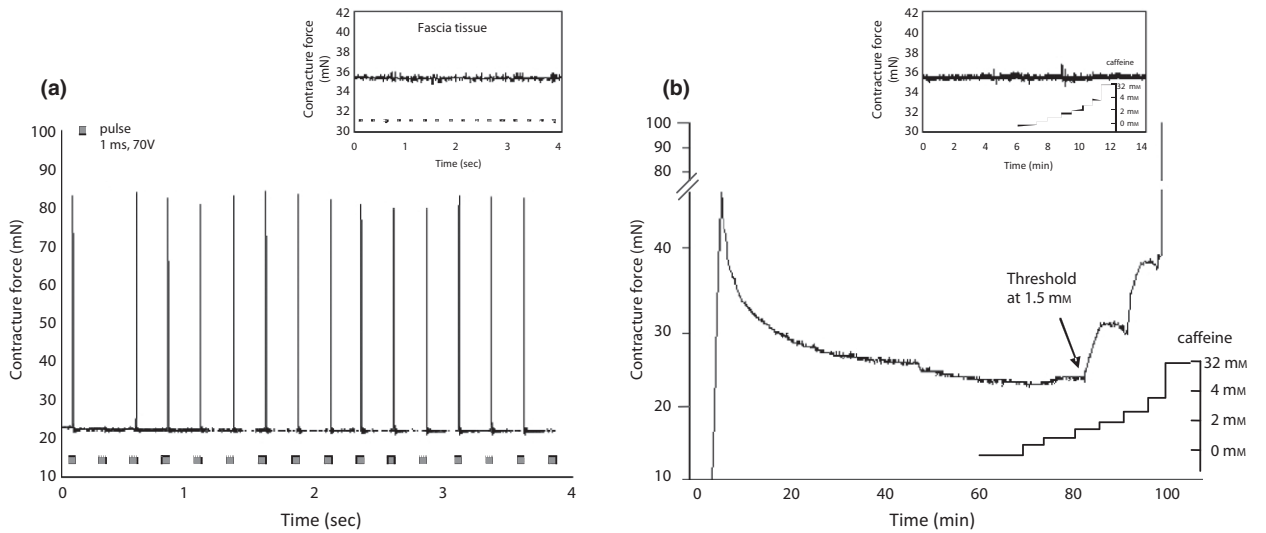

Figure 4 Responses of muscle to electrical and pharmacological stimulation. (a) Human muscle biopsies were exposed to 5 Hz and 20 Hz (5 Hz shown) stimulation resulting in muscle twitching. The inset chart on the upper right side shows no fascial tissue reaction to electrical stimulation. (b) Typical response of MH-susceptible human muscle specimen after caffeine challenge from 0.5 to 32 mM. The initial spike is due to pre-stretching of the specimen for optimum alignment of contractile filaments. Caffeine is added after a steady state is reached. The threshold concentration for a significant contracture, i.e. > 2 mN, is found at 1.5 mM caffeine. The inset chart on the upper right side shows the response of fascial tissue to caffeine challenge. No increase in force was detected.

no significant contraction of fascial tissue, suggesting that these receptors are not involved in myofibroblast contractile properties.

Other in-vitro studies support our hypothesis that contractility of myofibroblasts is mostly independent of increased cytosolic Ca^{2+} levels [17–19]. Although cyclic and rapid micro-contractile events of about 400 nm correlate with periodic Ca^{2+} oscillations, high isometric tension of myofibroblasts is predominantly mediated by inactivation of myosin light chain phosphatase via the Rho kinase, finally leading to sustained contracture [20, 21]. Rho kinase is upregulated in inflammatory conditions, thereby influencing myofibroblast contractility [22, 23]. Both drugs, effective on fascial tension, nitric oxide and mepyramine, are substances interfering with cytokine pathways involved in inflammatory processes [24, 25]. This suggests the inflammation cascade as a potential target to influence fascial tissue-associated pathology symptoms.

Finally, this study suggests that smooth muscle-like cellular contractions of myofibroblasts are able to influence human biomechanics and may complicate a wide range of neuromuscular symptoms, possibly providing new targets to alleviate the clinical symptomatic of such syndromes [26].

Based on this finding, varying amounts of connective tissue influence muscle force measurements with electrical or pharmacological stimulation. Although contractions of myofibroblasts might at least in part be mediated by increased Ca^{2+} levels, our results suggest that the underlying mechanism is independent of ryanodine receptors activation.

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Competing interests

No external funding and no competing interest declared.

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