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Preserved Ca²⁺ handling and excitation-contraction coupling in muscle fibers from diet-induced obese mice

¹Francisco Jaque-Fernandez, ²Agathe Beaulant, ¹Christine Berthier 0000-0002-9608-643X, ¹Laloé Monteiro, ¹Bruno Allard 0000-0002-7131-7828, ³Mariana Casas 0000-0002-6930-311X, ²Jennifer Rieusset 0000-0002-1587-2253, ¹Vincent Jacquemond 0000-0003-4944-270X

¹Univ Lyon, Université Claude Bernard Lyon 1, CNRS UMR-5310, INSERM U-1217, Institut NeuroMyoGène, 8 avenue Rockefeller, 69373 Lyon, France.

² Univ Lyon, CarMeN Laboratory, INSERM, INRA, INSA Lyon, Université Claude Bernard Lyon 1, 69310, Pierre-Bénite, France.

³Centro de Estudios Moleculares de la Célula, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

Running title: Muscle Ca²⁺ signaling in diabetes

The first detailed investigation of Ca²⁺ handling and excitation-contraction coupling in #skeletal muscle of a mouse model of #obesity. A work from @jacquemondlab at @iNeuroMyoGene and collaborators from @CarMeN_lab_Lyon and @IBiomedicas.

Corresponding author :
Vincent Jacquemond
Institut NeuroMyoGène
UMR CNRS 5310 - INSERM U1217 - Université Claude Bernard Lyon 1
Faculté de Médecine et de Pharmacie
8, Avenue Rockefeller, 69373 Lyon, France
Tel. (33) 4 26 68 82 69
E-mail : vincent.jacquemond@univ-lyon1.fr
ORCID iD : 0000-0003-4944-270X

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Abstract

Aims/hypothesis

Disrupted intracellular Ca^{2+} handling is known to play a role in diabetic cardiomyopathy but it has also been postulated to contribute to obesity- and type 2 diabetes-associated skeletal muscle dysfunction. Still, there is so far very limited functional insights into whether, and if so how, muscular Ca^{2+} homeostasis is affected in this situation so as to potentially determine or contribute to muscle weakness. In differentiated muscle, force production is under the control of the excitation-contraction coupling process: upon plasma membrane electrical activity, the $\text{Ca}_v1.1$ voltage-sensor/ Ca^{2+} channel in the plasma membrane triggers opening of the ryanodine receptor Ca^{2+} release channel in the sarcoplasmic reticulum membrane. Opening of the ryanodine receptor triggers the rise in cytosolic Ca^{2+} that activates contraction while Ca^{2+} uptake by the sarcoplasmic reticulum ATPase Ca^{2+} -pump promotes relaxation. These are the core mechanisms underlying the tight control of muscle force by neuronal electrical activity. This study aimed at characterizing their inherent physiological function in a diet-induced mouse model of obesity and type 2 diabetes.

Methods

Intact muscle fibers were isolated from mice fed either with a standard diet or with a high-fat, high-sucrose diet generating obesity, insulin resistance and glucose intolerance. Properties of muscle fibers were investigated with a combination of whole-cell voltage-clamp electrophysiology and confocal fluorescence imaging. The integrity and density of the plasma membrane network (transverse tubules) that carries the membrane excitation throughout the muscle fibers was assessed with the dye di-8-anepss. $\text{Ca}_v1.1$ Ca^{2+} channel activity was studied by measuring the changes in current across the plasma membrane elicited by voltage-clamp depolarizing pulses of increasing

amplitude. SR Ca^{2+} release through ryanodine receptors was simultaneously detected with the Ca^{2+} -sensitive dye rhod-2 in the cytosol. $\text{Ca}_v1.1$ voltage-sensing activity was separately characterized from the properties of intra-plasma-membrane charge movement produced by short voltage-clamp depolarizing pulses. Spontaneous Ca^{2+} release at rest was assessed with the Ca^{2+} -sensitive dye fluo-4. The rate of SR Ca^{2+} uptake was assessed from the time-course of cytosolic Ca^{2+} recovery after the end of voltage excitation using the Ca^{2+} -sensitive dye fluo-4FF. The response to a fatigue-stimulation protocol was determined from the time-course of decline of the peak fluo-4FF Ca^{2+} transients elicited by 30 trains of 5 ms-long depolarizing pulses delivered at 100 Hz.

Results

The transverse tubule network architecture and density were well preserved in the fibers from the obese mice. The $\text{Ca}_v1.1$ Ca^{2+} current and voltage-sensing properties were also largely unaffected with mean values for maximum conductance and maximum amount of charge of 196 ± 13 S/F and 32.9 ± 2 nC/ μF as compared to 234 ± 12 S/F and 30.7 ± 1.6 nC/ μF in fibers from mice fed with the standard diet, respectively. Voltage-activated SR Ca^{2+} release through ryanodine receptors also exhibited very similar properties in the two groups with mean values for maximum rate of Ca^{2+} release of 76.0 ± 6.5 and 78.1 ± 4.4 $\mu\text{M} \cdot \text{ms}^{-1}$, in fibers from control and obese mice, respectively. The response to a fatigue protocol was also largely unaffected in fibers from the obese mice, and so were the rate of cytosolic Ca^{2+} removal and the spontaneous Ca^{2+} release activity at rest.

Conclusions/interpretation

The functional properties of the main mechanisms involved in the control of muscle Ca^{2+} homeostasis are well preserved in muscle fibers from obese mice, at the level of both the plasma membrane and of the sarcoplasmic reticulum. We conclude that intracellular Ca^{2+} handling and

excitation-contraction coupling in skeletal muscle fibers are not primary targets of obesity and type 2 diabetes.

Keywords

Obesity, type 2 diabetes, skeletal muscle, Ca²⁺ homeostasis, excitation-contraction coupling, ryanodine receptor, sarcoplasmic reticulum Ca²⁺ release, cytosolic Ca²⁺ clearance.

Abbreviations

ECC Excitation-contraction coupling

FDB flexor digitorum brevis

GTT Glucose tolerance test

HFHSD High-fat, high-sucrose diet

ITT Insulin tolerance test

RYR1 Type 1 ryanodine receptor

SD Standard diet

SERCA Sarco/endoplasmic reticulum Ca-ATPase

SR Sarcoplasmic reticulum

T-tubule Transverse tubule

Research in context

What is already known about this subject?

- Obesity and type 2 diabetes are associated with skeletal muscle dysfunction
- High-fat diet induces a decrease in specific muscle force production

What is the key question?

- Does impaired Ca^{2+} homeostasis contribute to skeletal muscle dysfunction and weakness in obesity and type 2 diabetes?

What are the new findings?

- Ca^{2+} entry and voltage-sensing steps of excitation-contraction coupling at the plasma membrane level are unaffected in muscle fibers from obese mice.
- Properties of intracellular Ca^{2+} handling by the sarcoplasmic reticulum are also completely preserved in muscle fibers from obese mice.

How might this impact on clinical practice in the foreseeable future?

- The Ca^{2+} handling capacities of skeletal muscle that allow control of force production are intact in obesity and type 2 diabetes. These findings are important for our understanding of muscle function/dysfunction in these situations and may impact clinical practice in relation with physical exercise in patients.

Introduction

Obesity and its deleterious complications, insulin resistance and type 2 diabetes mellitus, are associated with skeletal muscle dysfunction [1,2]. Long-term systemic impairments related to the disease status such as cardiomyopathy and peripheral neuropathy and vascular disease eventually contribute but there is also evidence that the muscle tissue itself suffers from inherently reduced performance. Still, underlying mechanisms remain unclear and the situation is also complicated by concomitant processes operating to adapt muscle function to overweight-induced increased force demand. The literature related to altered muscle function in obesity and diabetes is intricate because of the diversity of experimental conditions and animal models used to tackle this issue, including a variety of genetically-modified murine models, selected murine strains, and pharmacological- or dietary-induction protocols [3,4]. If one focusses on the most common reason for obesity and type 2 diabetes in human, diet-induced obesity in animal models may be considered of somewhat more specific interest. Accordingly, there is reproducible evidence that a sufficiently long period (≥ 10 weeks) of high-fat diet induces a decrease in specific force production, as assessed *ex vivo* from mouse muscle [5-7].

Altered intracellular Ca^{2+} handling contributes to obesity-induced cardiac dysfunction [8,9] [see for review 10,11]. Skeletal muscle function critically depends upon stringent control of intracellular Ca^{2+} with three proteins playing a key role in this process: $\text{Ca}_v1.1$, a voltage sensor and Ca^{2+} channel in the t-tubule membrane, RYR1 the Ca^{2+} release channel in the SR membrane and SERCA the SR Ca^{2+} ATPase ensuring Ca^{2+} uptake and consequent cytosolic Ca^{2+} clearance. Muscle force is triggered by cytosolic Ca^{2+} increase, promoting Ca^{2+} binding to troponin C. This occurs when t-tubule depolarization sensed by $\text{Ca}_v1.1$ is transduced into opening of RYR1 channels and consequent SR Ca^{2+} release: the ECC process [12-15]. Besides activation of force, intracellular Ca^{2+} plays also a key role in other aspects of muscle function including metabolism and signaling

cascades involved in gene expression [16,17], processes that may also be modified because of altered Ca^{2+} homeostasis. Altogether, there is thus strong interest to determine whether functional aspects of Ca^{2+} homeostasis are affected in diet-induced obesity. Surprisingly, this has been largely experimentally overlooked so far, at least at the level of intact muscle fibers under membrane voltage control, a condition made essential by the stringent command exerted by plasma membrane voltage on intracellular Ca^{2+} homeostasis. In other words, no quantitative characterization of the functional features of Ca^{2+} handling at the physiological milliseconds-time range of activation of ECC is available in this disease situation. This is at odds with the fact that potential mechanisms involved in Ca^{2+} dysregulation and target mechanisms have been largely highlighted in the literature [2,18].

We have used single muscle fibers isolated from mice fed with a HFHSD generating obesity, hyperinsulinemia, insulin resistance and glucose intolerance [19] to perform an extensive functional characterization of intracellular Ca^{2+} signaling and ECC in that situation.

Methods

All experiments and procedures were performed according to the ethics principles of the French Department of Veterinary Services and the French Ministry for Higher Education, Research and Innovation, in accordance with the guidelines of the local animal ethics committee of the University Claude Bernard Lyon 1, the French Ministry of Agriculture (decree 87/848), and the revised European Directive 2010/63/EU. Experiments were performed at room temperature (20–22°C).

HFHSD-induced diabetic mice

The experimental protocol was approved by the Animal Experimentation Committee # C2EA-15 of the Rhône-Alpes Region. 4-week-old C57BL/6J01aHsd male mice were purchased from ENVIGO (Gannat, France) and housed at 22°C with a 12-h light/ dark cycle. Following delivery, mice were left for 1 week to adapt to the new environment and then randomly separated in 2 groups: one with free access to a standard chow diet (SD) (Rod16-A, Genobios: 16.9 % proteins, 4.3 % lipids) and the other with free access to a pelleted HFHSD diet (260HF U8978 version 19; SAFE; 20 % proteins, 36 % lipids, 35 % carbohydrates) for 16 weeks as described previously [19]. For simplicity, mice, and fibers isolated from the mice in the two groups, are referred to as SD and HFHSD. Two sets of 5 mice of each group were used in this study. The first set was dedicated to characterization of t-tubule membrane architecture, voltage-activated Ca^{2+} current and intracellular Ca^{2+} transients, and spontaneous Ca^{2+} release events at rest. In the second set we measured intramembrane charge movement and spontaneous Ca^{2+} release events. In the first group, body mass and blood glucose were assessed twice a week and a glucose tolerance test (GTT) and an insulin tolerance test (ITT) were performed during weeks # 4, 8, 12 and 16 post-beginning of the diet, as previously described [19]. In the second group an ITT and a GTT test were performed during week #15. In all mice, body weight and blood glucose were assessed before sacrifice.

Preparation of isolated muscle fibers

Single fibers from the *flexor digitorum brevis* (FDB) and interosseus muscles were isolated as previously described [20]: mice were anaesthetized with isoflurane and killed by cervical dislocation before removal of the muscles. Muscles were incubated in Tyrode containing 2 mg/ml collagenase (Sigma, type 1) for 60 min at 37°C. Single fibers were obtained by mechanical

trituration within a culture μ -dish (Ibidi, Planegg / Martinsried, Germany) filled with culture medium containing 10% fetal bovine serum (MI199; Eurobio, France), the bottom of which had been first coated with a thin layer of silicone. Isolated fibers settled on the silicone surface and were then embedded with additional silicone so that only a portion of the fiber extremity remained in contact with the extracellular medium. The initial silicone coating of the chamber bottom was necessary because of the hydrophobic nature of the silicone grease which makes it very hard to adhere to a solid substrate in a liquid environment. The silicone grease we used was SILBIONE Paste 70428 (Elkem Silicones, Saint-Fons, France).

Electrophysiology

Voltage-clamp experiments were performed on FDB muscle fibers using a micropipette filled with an intracellular-like medium (see Solutions). The pipette tip was inserted into the silicone-insulated part of the fiber and was gently crushed against the chamber bottom to ease dialysis and reduce series resistance. Composition of the extracellular solution and of the pipette solution were adapted to the experiment type (see Solutions). The fiber interior was dialyzed for 30 min to allow for intracellular equilibration of the solution before starting measurements. The chlorided silver wire inside the pipette was connected to an RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) in whole-cell configuration, in combination with an analog-digital converter (Digidata 1440A, Axon Instruments, Foster City, CA) controlled by pClamp 9 software (Axon Instruments). The effective series resistance was further decreased by analog compensation. Unless otherwise specified, the holding voltage was set to -80 mV.

$\text{Ca}_v1.1$ Ca^{2+} current was measured in response to 0.5 s-long depolarizing steps. The linear leak component of the current was removed by subtracting the adequately scaled value of the current

measured during a -20 mV step. This subtraction did not fully eliminate the outward current measured during pulses to values between -50 and -30 mV, below the threshold of activation of the Ca²⁺ current. In order to correct for this, the voltage dependence of the values for the residual current was fitted by a linear function from -50 to -30 mV; the fit was extrapolated to the entire range of voltage values and fitted values were subtracted from the measured values for peak Cav1.1 current. The voltage dependence of peak current values (normalized to the capacitance), was fitted with the following equation:

$$I(V) = G_{max}(V-V_{rev})/(1+\exp((V_{0.5}-V)/k))$$

with $I(V)$ the peak current density at the command voltage V , G_{max} the maximum conductance, V_{rev} the apparent reversal potential, $V_{0.5}$ the half-activation potential and k the steepness factor.

Cav1.1 charge movement currents were measured from a holding voltage of -100 mV and analyzed as previously described [21,22]. In brief, adequately scaled control records elicited by 25 ms-long pulses of -20 mV were subtracted from the current elicited by test depolarizing pulses of same duration to various levels [23]. The amount of charge moved during a test pulse was measured by integrating the *on* and *off* portions of the corrected test current records. The steady-state distribution of charge (normalized to fiber capacitance) was fitted with a two-state Boltzmann function:

$$Q(V)=Q_{max}/(1+\exp[(V_{0.5}-V)/k])$$

with Q_{max} the maximal available charge, $V_{0.5}$ the voltage of equal charge distribution and k the steepness factor.

Confocal imaging

Confocal imaging was conducted with a Zeiss LSM 5 Exciter equipped with a 63× oil immersion objective (numerical aperture 1.4). For detection of rhod-2 and fluo-4 FF fluorescence, excitation

was from the 543 nm line of a HeNe laser and from the 488 nm line of an Argon laser, respectively, and fluorescence was collected above 560 nm and above 505 nm, respectively. Rhod-2 and fluo-4 FF fluorescence changes were imaged using the line-scan mode (x,t) of the system and expressed as F/F_0 where F_0 is the baseline fluorescence. Quantification of the Ca^{2+} release flux underlying the rhod-2 Ca^{2+} transients was performed as previously described [24]. In each fiber, the voltage-dependence of the peak rate of Ca^{2+} release was fitted with a Boltzmann function.

Di-8-anepps and fluo-4 fluorescence were collected above 505 nm with 488 nm excitation. For imaging the t-tubule network, interosseus muscle fibers were incubated for 30 minutes in the presence of 10 μM di-8-anepps. The t-tubule density was estimated as described previously [24].

For Ca^{2+} sparks measurements, interosseus muscle fibers were incubated for 30 minutes in the presence of 10 μM fluo-4 AM. 30 consecutive confocal frames of fluo-4 fluorescence ($102.4 \times 102.4 \mu\text{m}$, 989 ms per frame) were acquired in each fiber. To quantify the Ca^{2+} sparks activity, images in the stack were smoothed and the standard deviation of fluorescence intensity at each pixel position, along the stack, was calculated. The 20 % largest values in the standard deviation image were removed to calculate the mean standard deviation of silent areas. Active area was then defined as pixels positions exhibiting at least 1.5 times larger values of standard deviation than the mean standard deviation value from silent areas.

Buffers and incubation media

Tyrode solution contained (in mmol/l): 140 NaCl, 5 KCl, 2.5 CaCl_2 , 2 MgCl_2 , 10 HEPES. The extracellular solution for measurements of $\text{Ca}_v1.1$ Ca^{2+} current and Ca^{2+} transients contained (in mmol/l) 140 TEA-methane-sulfonate, 2.5 CaCl_2 , 2 MgCl_2 , 1 4-aminopyridine, 10 HEPES and

0.002 tetrodotoxin. For measurements of fluo-4 FF Ca²⁺ transients, it also contained 0.05 N-benzyl-p-toluene sulfonamide (BTS) to block contraction. The pipette solution contained (in mmol/l) 120 K-glutamate, 5 Na₂-ATP, 5 Na₂-phosphocreatine, 5.5 MgCl₂, 5 glucose, 5 HEPES. For measurements of rhod-2 and fluo-4 FF Ca²⁺ transients it also contained 15 EGTA, 6 CaCl₂, 0.1 rhod-2, and 0.1 fluo-4 FF, respectively.

For measurements of Cav1.1 charge movement the extracellular solution contained (in mmol/l) 140 TEA-methane-sulfonate, 0.1 CaCl₂, 3 MgCl₂, 1 CdCl₂, 0.5 MnCl₂, 1 4-aminopyridine, 0.5 9-anthracene-carboxylic acid, 10 HEPES and 0.002 tetrodotoxin and fibers were dialyzed through the pipette with a solution containing (in mmol/l) 140 TEA-methane-sulfonate, 5 Na₂-ATP, 5 Na₂-phosphocreatine, 5.5 MgCl₂, 5 glucose, 5 HEPES. BTS was purchased from Tocris Bioscience (Bio-Techne, Lille, France). All fluorescent indicators were purchased from Thermo Fisher Scientific (Illkirch, France). All solutions were adjusted to pH 7.20.

Statistics

Statistical analysis was performed with Origin 8.0. Data values are presented as means ± SEM for *n* fibers. Statistical significance was determined using a Student's t-test (*P≤0.05).

Results

Obesity, insulin resistance and glucose intolerance in the HFHSD mice

The status of our animals over the course of the diet protocol is recapitulated in Fig. 1, as assessed from the first group of mice (see Research design and methods). At the end, the body weight of the HFHSD animals was ~1.7 times that of SD ones (Fig. 1a); from the 2 groups of mice used in this

study, mean values for end-body weight of SD and HFHSD animals were 28.6 ± 0.5 (n=9) and 50.1 ± 0.9 g (n=9, $P < 0.0001$), respectively. There was no associated change in the weight of the gastrocnemius (0.17 ± 0.01 , n=9 and 0.21 ± 0.02 g, n=9, $P = 0.14$) nor of the heart (0.17 ± 0.01 , n=9 and 0.21 ± 0.02 g, n=9, $P = 0.09$), whereas the weight of the liver (1.30 ± 0.04 , n=9 and 3.11 ± 0.34 g, n=9, $P < 0.0001$) and of the white adipose tissue (measured as the epididymal adipose tissue: 0.51 ± 0.04 , n=9 and 2.06 ± 0.17 g, n=9, $P < 0.0001$) were substantially increased in HFHSD *versus* SD animals, respectively. Results from glucose and insulin tolerance tests performed at weeks # 4, 8, 12 and 16 are shown in Fig. 1b and Fig. 1c, respectively: HFHSD mice maintained a more elevated blood glucose level following injection of either glucose or insulin: from the 10-minutes time point following either glucose or insulin injection, the mean glucose level in HFHSD mice ranged between approximately 1.5 and 2.3 times the level in SD mice, (the difference being statistically significant between the two groups at any time point of the 4 sets of measurements), establishing the glucose intolerance and the systemic insulin resistance, and thus the diabetic status generated by the protocol.

T-tubule network and passive electrical properties in SD and HFHSD muscle fibers

The t-tubule membrane system plays a critical role in ECC and in glucose transport and high-fat diet may enhance its cholesterol content [25] with potential adverse consequences for t-tubules integrity and function [26,27]. We labelled the plasma membrane of fibers with di-8-anepps. Corresponding *x,y* confocal images from SD and HFHSD muscle fibers (Fig. 2a) showed no sign of alteration of the network in the HFHSD group and the transverse profile of fluorescence showed the expected double-peak pattern characteristic of t-tubule organization in mammalian muscle (bottom graphs in Fig. 2a). Analysis of di-8-anepps images collected from 20 muscle fibers from 2 SD mice and from 30 fibers from 3 HFHSD mice, showed no change in t-tubule density and,

incidentally, but, incidentally, the mean value for sarcomere length was slightly but significantly reduced in the HFHSD as compared to the SD group (2.04 ± 0.01 vs 2.11 ± 0.02 , $P=0.001$, Fig. 2b). . Preservation of the t-system in the HFHSD fibers was confirmed by the passive electrical properties of the fibers: membrane current records from fibers used to measure $\text{Ca}_v1.1 \text{ Ca}^{2+}$ current gave mean values for capacitance and input resistance of 949 ± 68 and 930.6 ± 61 pF, and 11.9 ± 1 and $12.5 \pm 0.8 \text{ M}\Omega$, in the SD group (n=21 fibers from 4 mice) and in the HFHSD group (n=30 fibers from 5 mice), respectively. Because mean values for fiber diameter did not differ between the SD ($44.8 \pm 1.4 \mu\text{m}$) and HFHSD group ($43.4 \pm 1.4 \mu\text{m}$) and assuming that a fiber portion of always similar length length was always under voltage-clamp, results establish that the t-tubule network is preserved in the obesity situation.

Ca_v1.1 Ca²⁺ current and voltage-sensing properties in SD and HFHSD muscle fibers

$\text{Ca}_v1.1$ is the most well-identified and characterized Ca^{2+} entry pathway across the plasma/t-tubule membrane of muscle fibers. Example $\text{Ca}_v1.1 \text{ Ca}^{2+}$ current records from a SD and from a HFHSD muscle fiber are shown in Fig. 3a; mean values for peak Ca^{2+} current *versus* voltage in the two groups are shown in Fig. 3b whereas mean values for the parameters obtained from fitting each individual peak current *vs* voltage relationship are shown in Fig. 3c. There was no significant change in any parameter in the HFHSD group.

$\text{Ca}_v1.1$ also exerts the critical function of coupling the t-tubule depolarization to RYR1 Ca^{2+} channels activation in the SR membrane. $\text{Ca}_v1.1$ voltage-sensing function was assessed from intramembrane charge movement. Example traces of charge current from a SD and from a HFHSD muscle fiber are shown in Fig. 4a whereas mean values for the amount of charge *versus* voltage are shown in Fig. 4b (data from 22 SD fibers and 21 HFHSD fibers, each from 3 mice in each group). Properties of charge movement were similar in SD and HFHSD muscle fibers,

demonstrating that the amount of Cav1.1 in the plasma/t-tubule membrane and its voltage sensing properties are preserved in this situation.

Voltage-activated SR Ca²⁺ release

Properties of RYR1 channels activity upon fiber activation were assessed from voltage-clamp activated cytosolic Ca²⁺ transients. A family of such F/F₀ rhod-2 transients from each group is shown in Fig. 5a while traces for the corresponding calculated rate of SR Ca²⁺ release are shown in Fig. 5b. As classically reported in this preparation, SR Ca²⁺ release exhibits an early peak followed by a decay towards a much lower level. There was no obvious qualitative feature of the rhod-2 transients and of the rate of Ca²⁺ release that differed between SD and HFHSD fibers. This was confirmed by the analysis showing that neither mean values for peak rate of SR Ca²⁺ release (peak d[Ca_{Tot}]/dt), nor for its time to peak (t_{peak}) nor for total amount of released Ca²⁺ ([Ca_{Tot}] calculated from the time integral of the Ca²⁺ release trace) statistically differed between the two groups (Fig. 5c). Thus, the physiological RYR1 channel activity and the SR Ca²⁺ content are preserved in HFHSD fibers.

Cytosolic Ca²⁺removal capabilities and response to a fatiguing stimulation protocol

Functional impact of HFD-induced obesity on SERCA activity may occur because of altered SR phospholipid composition [28,29] and/or changes in expression or efficiency of the SERCA regulator sarcolipin [30]. SERCA is the major actor of cytosolic Ca²⁺ clearance. We assessed the cytosolic removal capabilities of fibers by measuring the time constant of the Ca²⁺ signal decay following repolarization-induced Ca²⁺ release termination. For this we used the intermediate affinity Ca²⁺-sensitive dye fluo-4FF under non EGTA-buffering conditions of the intracellular medium (see Methods). Illustrative fluo-4FF Ca²⁺ transients elicited by maximum activation of SR

Ca^{2+} under these conditions are presented in Fig. 6a. In each fiber tested, a single exponential function was fitted to the declining phase of the transients. Corresponding mean values for the time constant of decay showed no sign of any trend of difference between the two groups (Fig. 6b, data from 8 fibers from 4 SD mice and from 13 fibers from 5 HFHSD mice).

Under the same conditions, we explored the response of the fibers to an exhausting stimulation protocol consisting in consecutive trains of 30, 5-ms-long, pulses from -80 mV to +60 mV delivered at 100 Hz. 30 trains were applied, separated by a 0.7 s interval. This protocol produces a rapid reduction of SR Ca^{2+} release as illustrated in Fig. 7a which shows examples of the first and last fluo-4FF transients recorded from a fiber of each group, in response to a full protocol. Fig. 7b shows the entire sequence of fluo-4FF transients from the two same fibers over time. Such records were collected from 8 fibers from 4 SD mice and from 12 fibers from 5 HFHSD mice, respectively. Mean values for the normalized time course of decline of the peak F/F_0 transients in the two groups are presented in Fig. 7c (left). In each fiber, the time course was fitted by a single exponential plus constant function: corresponding mean values (Fig. 7c, right) establish that there was no stringent alteration in HFHSD fibers. The time constant of decay was increased by a factor of ~ 1.25 in the HFHSD group, but the difference did not reach statistical significance ($P=0.057$).

Spontaneous Ca^{2+} release activity at rest

Spontaneous activity of RYR1 channels at rest under the form of local SR Ca^{2+} release events called Ca^{2+} sparks, is a hallmark of several muscle disease situations [24,31,32]. We tested whether this was the case in HFHSD fibers using confocal imaging of the Ca^{2+} -sensitive dye fluo-4: x,y images of the standard deviation of fluo-4 fluorescence intensity along 30 consecutive confocal frames (total time of ~ 30 s) from a SD fiber and from a HFHSD fiber are shown in Fig. 8a: SD

fibers were basically devoid of Ca^{2+} sparks activity whereas some HFHSD fibers did exhibit a few sparks. Ca^{2+} sparks activity was quantified in 28 muscle fibers from 3 SD mice and from 46 fibers from 5 HFHSD mice, respectively. The left graph in Fig. 8b shows the mean value for relative fiber area exhibiting Ca^{2+} sparks activity over a period of ~30 s (see Methods) in the two group, while the graph on the right shows the corresponding distribution of the number of fibers according to this parameter. The mean fiber area exhibiting Ca^{2+} sparks activity was significantly ($P=0.043$) increased in the HFHSD group but in a quantitatively very modest manner (0.6 % of the fiber area as compared to 0.4 % in the SD group), this being essentially due to 4 muscle fibers issued from one HFHSD mouse, exhibiting an active relative fiber area larger than 1.5 %.

Discussion

Altered skeletal muscle function associated with obesity and type 2 diabetes is a major burden for patient's mobility and locomotion [e.g. 33]. In the long run, muscle-extrinsic mechanisms related to cardiac and vascular complications obviously play a role and so does muscle atrophy. However, there is also evidence that muscle-intrinsic impaired force production is involved [2] but the underlying mechanisms remain obscure. HFD-induced obesity in mouse is associated with reduced specific muscle force, prompting the interest of this model to decipher the muscle-intrinsic mechanisms. Intracellular Ca^{2+} plays a key role in numerous aspects of muscle function including triggering contraction. In order to ensure this particular function, intracellular Ca^{2+} concentration has to remain under tight control and, accordingly, there are numerous examples of muscle dysfunction due to altered Ca^{2+} handling and ECC [see 34-37].

Defective Ca^{2+} regulation was early suggested to occur in a pharmacologically-induced diabetes model and in a genetically obese mouse model: specifically, accumulation of Ca^{2+} together with increased amount of Cav1.1 was shown in skeletal muscle from a rat model of streptozocin-induced diabetes [38] and Ca^{2+} regulation was reported abnormal in the *ob/ob* mouse model of obesity, contributing to premature fatigue [39]. If referring specifically to HFD models, altered Ca^{2+} handling is suggested to play a role in obesity-induced muscle dysfunction through HFD-induced increased production of reactive oxygen species (ROS) which would promote SR Ca^{2+} leak through enhanced S-nitrosylation of RYR1 channels [40] and possibly also, impair SERCA function [41,42]. In addition, altered expression and/or efficiency of sarcolipin [30] could also contribute to impair cytosolic Ca^{2+} clearance with potentially consequences for muscle metabolism and energy expenditure through activation of Ca^{2+} signaling pathways [43,44]. Still, altogether, functional correlates for these alterations in terms of Ca^{2+} handling within the course of physiological ECC are poorly documented.

The strength of the present study is to achieve the first detailed quantitative investigation of the functional properties of intracellular Ca^{2+} handling and ECC in a diet-induced mouse model of obesity, using single isolated muscle fibers under voltage control. Our results demonstrate remarkably well preserved Ca^{2+} handling capabilities in terms of voltage-dependent Ca^{2+} entry across the plasma membrane through Cav1.1, Cav1.1 properties as ECC voltage-sensors, voltage-activated SR Ca^{2+} release through RYR1 channels, SERCA-mediated cytosolic Ca^{2+} removal and resistance to a fatiguing stimulation protocol. Results concur with data from Eshima et al. [6] suggesting that HFD elicits no change in the expression levels of calcium handling-related proteins, but they further prove that the physiological operating function of these proteins during ECC is largely unaffected in that situation. They also concur with results from Bruton et al [39] showing

that resting and tetanic Ca^{2+} under unfatigued conditions are similar in *ob/ob* and wild-type muscle fibers. Overall, our results do not exclude that the previously suggested, above-mentioned, alterations of Ca^{2+} handling do occur, but prove that they remain very minor in terms of functional impact on the basic function of ECC and are thus likely not directly involved in chronic reduced muscle performance. For instance, the fact that some muscle fibers from HFHSD mice exhibit an enhanced propensity for spontaneous Ca^{2+} release at rest may be related to the presence of S-nitrosylated leaky RYR1 channels, but overall, this clearly does not compromise the SR Ca^{2+} content and proper function of the Ca^{2+} release machinery. It may then be that the reported alterations of Ca^{2+} homeostasis remain silent in regard to ECC function but relevant in regard to activation of specific Ca^{2+} -dependent signaling pathways. Importantly also, the preserved Ca^{2+} handling capabilities in isolated fibers maintained under our controlled experimental conditions does not eliminate the possibility that the situation differs *in vivo*, due to the specific environment experienced by the fibers in the diseased organism. It will thus be of high interest in the future to investigate the function of ECC and Ca^{2+} handling in isolated fibers challenged by conditions mimicking this environment, including incubation of the fibers in the presence of insulin and glucose. Along this line for instance, it is quite stimulating that elevated glucose was recently reported to alter t-tubule morphology of isolated muscle fibers (26).

In summary, our results demonstrate that the intrinsic function of intracellular Ca^{2+} signaling and excitation-contraction coupling is amazingly well preserved in muscle fibers from a mouse model of obesity and type 2 diabetes.

Acknowledgements

Data availability

Original datasets and datasets generated by the described analysis procedures are available from the corresponding author upon reasonable request. Critical resources supporting the results are also available upon reasonable request.

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Authors' relationships and activities

The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement

V.J., J.R. and F.J. conceived and coordinated the study. J.R. and A.B. generated the mouse model and collected metabolic data. F.J. conducted the electrophysiological and fluorescence experiments. F.J., L.M. and V.J. performed the main electrophysiological and fluorescence data analysis. All authors made critical contributions to data analysis, interpretation and discussion and to manuscript preparation, and approved the final version. V.J. and J.R. wrote the manuscript.

V.J. is the guarantor of this work and, as such, accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Figure legends

Figure 1 – Body mass and glucose and insulin tolerance tests in SD and HFHSD mice. (a) Mean values for body weight of SD (filled circles, n=9) and HFHSD mice (open circles, n=9), over the course of the diet protocol. (b, c), Mean values for blood glucose concentration before (t=0) and after intraperitoneal injection of either 2 mg/g glucose (b) or 0.75 units/kg insulin (c). Measurements were performed at weeks # 4, 8, 12 and 16 of the protocol.

Figure 2 – T-tubule system network. (a) x,y confocal images of di-8-anneps fluorescence from separate SD (left) and HFHSD muscle fibers (right) at two different magnifications (top and bottom). Graphs at the bottom show the transverse pattern of di-8-anneps fluorescence over the highlighted region in the corresponding above x,y images. (b) Mean values for t-tubule density index (left) and for sarcomere length (right) in the SD (n=20 fibers from 2 mice) and HFHSD (n=30 fibers from 3 mice) groups.

Figure 3 – Cav1.1 Ca²⁺ current. (a) example traces of Ca²⁺ current in a SD (left) and in a HFHSD muscle fiber (right) in response to the voltage-clamp pulse protocol shown on top. (b) Mean values for the peak Ca²⁺ current density vs voltage in the SD (n=20 fibers from 4 mice) and HFHSD (n=29 fibers from 8 mice) groups. (c) Corresponding mean values for the parameters obtained from fitting each individual peak current vs voltage relationship with the appropriate function (see Methods).

Figure 4 – Cav1.1 charge movement. (a) example traces of Cav1.1 charge movement currents in a SD (left) and in a HFHSD muscle fiber (right) in response to voltage-clamp depolarizing pulses from -100 mV to values ranging between -80 and +20 mV. (b), Mean values for the amount of

Cav1.1 charge density *vs* voltage in the SD (n=22 fibers from 3 mice) and HFHSD (n=21 fibers from 3 mice) groups. Bar graphs on the right present the mean values for the parameters obtained from fitting each individual peak charge *vs* voltage relationship with the appropriate function (see Methods).

Figure 5 – Voltage-activated SR Ca²⁺ release. (a) Example traces of rhod-2 F/F₀ Ca²⁺ transients in a SD (left) and in a HFHSD muscle fiber (right) in response to the voltage-clamp pulse protocol shown on top. (b) Rate of SR Ca²⁺ release (d[Ca_{Tot}]/dt) calculated from the rhod-2 transients shown in (a). For the sake of clarity, the rate traces are only shown throughout the portion of rhod-2 F/F₀ traces highlighted by an arrow in (b). (c), Left, mean values for the peak rate of SR Ca²⁺ release (peak d[Ca_{Tot}]/dt) *vs* voltage in the SD (n=21 fibers from 4 mice) and HFHSD (n=30 fibers from 5 mice) groups. The two graphs on the right show corresponding values for the total amount of release Ca²⁺ ([Ca_{Tot}], calculated from the time integral of the rate) and for the time to peak SR Ca²⁺ release (t_{peak}).

Figure 6 – Cytosolic Ca²⁺ removal. (a) Fluo-4FF F/F₀ Ca²⁺ transients elicited by the voltage clamp pulse protocol shown on top in a SD muscle fiber (top) and in a HFHSD muscle fiber (bottom). (b), Mean values for the time constant of fluo-4FF fluorescence decay *vs* pulse duration in the SD (n=8 fibers from 4 mice) and HFHSD (n=13 fibers from 5 mice) groups.

Figure 7 – Response to a fatigue protocol. (a), Representative fluo-4FF fluorescence traces from a SD (left) and from a HFHSD muscle fiber (right) challenged by the voltage-clamp depolarization protocol shown on top, consisting of 5-ms-long pulses from -80 mV to +60 mV delivered at 100 Hz. This protocol was repeated 30 times: the two superimposed fluo-4FF traces correspond to the

responses to the first (largest) and to the last response of each fiber. **(b)**, Full time course of the fluo-4FF fluorescence transients over the course of the entire protocol (same muscle fibers as in **a**). **(c)**, Left, mean time course of the decay of the relative peak fluo-4FF Ca^{2+} transients over the full protocol. Right, mean values for the time constant of decay of the peak fluo-4FF transients and corresponding final level, obtained from a single exponential plus constant fit to the data in each fiber. Results are from 8 muscle fibers from 4 SD mice and from 12 muscle fibers from 5 HFHSD mice.

Figure 8 – Spontaneous Ca^{2+} release activity at rest. **(a)** Images of the standard deviation of fluo-4 fluorescence intensity in a SD and in a HFHSD muscle fiber, over the course of a series of 30 *x,y* confocal scans. **(b)** Left, mean values for the relative fiber area exhibiting spontaneous Ca^{2+} release in SD and HFHSD muscle fibers; right, corresponding distribution of the number of muscle fibers according to the relative active area.