Role of TRPC1 channel in skeletal muscle function

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Zanou N, Shapovalov G, Louis M, Tajeddine N, Gallo C, Van Schoor M, Anguish I, Cao ML, Schakman O, Dietrich A, Lebaq J, Ruegg U, Roulet E, Birnbaumer L, Gailly P. Role of TRPC1 channel in skeletal muscle function. Am J Physiol Cell Physiol 298: C149–C162, 2010. First published October 21, 2009; doi:10.1152/ajpcell.00241.2009.—Skeletal muscle contraction is orchestrated essentially by the release of Ca2+. In a previous study, we showed (24) that a decrease on the order of 30% of the Ca2+ content in the SR is sufficient to induce an entry of Ca2+ through the so-called store-operated channels. Interestingly, it has been found that a single action potential triggers the release of 0.2–0.3 mM Ca2+ from the SR into the cytoplasm (10), which corresponds to more than a quarter of the SR Ca2+ content (27). In addition, electrically stimulated isotonic contractions of soleus muscle led to a 30-fold increase of 45Ca2+ uptake compared with resting muscle (36). Thus it seems reasonable to assume that store-operated channels are indeed activated during physiological contractions.

In previous work, we have shown that store-operated channels are present in skeletal muscles and that their activity is abnormally increased in Duchenne muscular dystrophy (71), a myopathy due to the lack of dystrophin, a cytoskeletal protein, leading to a perturbation of Ca2+ homeostasis (30–32, 34, 58, 69). Using a RNA antisense strategy targeted to the transient receptor potential (TRP) box motif [EWKFA motif found at the COOH terminus, close to the last transmembrane domain in canonical TRP (TRPC) isoforms and modified in melastatin TRP (TRPM) and TRP vanilloid (TRPV) isoforms], we proposed that these channels, abnormally regulated in dystrophic fibers, might be constituted of TRP channel isoforms. In particular, TRPC1 was shown to be localized close to the plasma membrane and to interact with α-syntroph in that channel might constitute a component of store-operated channels. The role of TRPC1 in Duchenne muscular dystrophy might also involve its interactions with caveolin-3, Src kinase, and Homer proteins (Refs. 35, 67; reviewed in Ref. 1). Several reports propose that TRPC1 channel might constitute a component of store-operated channels (for review, see Refs. 6, 7, 11, 62), possibly associated with another channel called Orai1 (28, 44, 65), and with STIM1, an intrareticular Ca2+ sensor (7, 38, 47, 74, 75).

IT IS WELL KNOWN that adult skeletal muscle fibers do not exchange much Ca2+ with the extracellular medium during contraction. Their sarcoplasmic reticulum (SR) is rich in sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) pumps and has a high buffering capacity, mostly due to calsequestrin. Ca2+ extrusion through the plasma membrane is very slow (reviewed in Ref. 54). Therefore, almost all the Ca2+ released from the SR is rapidly restored after stimulation, and for that reason muscle fibers can twitch for hours in the absence of extracellular Ca2+ (8). However, evaluations of Ca2+ influx with the 45Ca2+ uptake technique indicate that each twitch contraction induces a small increase of Ca2+ entry (13).

In a previous study, we showed (24) that a decrease on the order of 30% of the Ca2+ content in the SR is sufficient to induce an entry of Ca2+ through the so-called store-operated channels. Interestingly, it has been found that a single action potential triggers the release of 0.2–0.3 mM Ca2+ from the SR into the cytoplasm (10), which corresponds to more than a quarter of the SR Ca2+ content (27). In addition, electrically stimulated isotonic contractions of soleus muscle led to a 30-fold increase of 45Ca2+ uptake compared with resting muscle (36). Thus it seems reasonable to assume that store-operated channels are indeed activated during physiological contractions.

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Using a RNA antisense strategy targeted to the transient receptor potential (TRP) box motif [EWKFA motif found at the COOH terminus, close to the last transmembrane domain in canonical TRP (TRPC) isoforms and modified in melastatin TRP (TRPM) and TRP vanilloid (TRPV) isoforms], we proposed that these channels, abnormally regulated in dystrophic fibers, might be constituted of TRP channel isoforms. In particular, TRPC1 was shown to be localized close to the plasma membrane and to interact with α-syntroph in that channel might constitute a component of store-operated channels. The role of TRPC1 in Duchenne muscular dystrophy might also involve its interactions with caveolin-3, Src kinase, and Homer proteins (Refs. 35, 67; reviewed in Ref. 1). Several reports propose that TRPC1 channel might constitute a component of store-operated channels (for review, see Refs. 6, 7, 11, 62), possibly associated with another channel called Orai1 (28, 44, 65), and with STIM1, an intrareticular Ca2+ sensor (7, 38, 47, 74, 75).

However, this is challenged by different reports showing that store-operated entry of Ca2+ is orchestrated essentially by STIM and Orai1 functioning independently of TRPC channels (Refs. 21, 50; for review, see Ref. 19). In skeletal muscle, this entry occurs essentially through the transverse tubule system, a situation that involves molecular agonists distributed throughout junctional membranes (12, 45).

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Recently, Lyfenko and Dirksen (50) showed that a Ca
 entry pathway distinct of the store-operated pathway exists in skeletal muscle (reviewed in Ref. 23). This entry is activated by depolarization. The molecular players have not yet been identified, but this pathway does not involve STIM1 and Orai1.

In this paper, we present new evidence in order to try to evaluate the real importance of TRPC1 channel in the influx of Ca
+ in skeletal muscle and to investigate its role in muscle function. We thus characterized Ca
+ currents and intracellular Ca
+ concentration ([Ca
+]i) transients as well as mechanical function in vitro and in vivo in TRPC1+/+ and TRPC1−/− mice.

MATERIALS AND METHODS

TRPC1+/+ and TRPC1−/− Mice

Generation of TRPC1−/− mice has been described previously (22). TRPC1−/− and TRPC1+/+ were obtained from heterozygous animals. TRPC1−/− were compared with their TRPC1+/+ control sex-matched littermates. TRPC1−/− mice were viable and fertile, with normal litter sizes compared with TRPC1+/+ mice. They appeared to be healthy, had a normal life span, and did not present any signs of major neurological disorder or metabolic disease.

Expression of TRPC Channels

For mRNA quantification, tibialis anterior, extensor digitorum longus (EDL), and soleus muscles were extracted with the Ribopure kit (Ambion) and reverse-transcribed with SuperScript II RNAse H (Invitrogen). Gene-specific PCR primers were designed with Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). To avoid amplification of genomic DNA, primers were chosen in different exons. All primers (Table 1) were purchased from Eurogentec. The cyclophilin D housekeeping gene and the genes of interest were amplified in parallel. Real-time RT-PCR was performed with 5 µl of cDNA, 12.5 µl of SYBR Green Mix (Bio-Rad), and each primer at 300 nM in a total reaction volume of 25 µl. The reaction was initiated at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 1 min, and extension at 72°C for 10 s.

Data were recorded on a MyiQ Real-Time PCR Detection System (Bio-Rad), and cycle threshold (Ct) values for each reaction were determined with analytical software from the same manufacturer. Each cDNA was amplified in duplicate, and Ct values were averaged for each duplicate. The average Ct value for cyclophilin D was subtracted from the average Ct value for the gene of interest, giving ΔCt (n = 5 or 6). Note that gene expression is inversely proportional to ΔCt. ΔCt values were obtained from TRPC1−/− samples (n = 4–6). ND, not detected.

Muscle Mechanical Measurements

Ten- to sixteen-week old TRPC1+/+ and TRPC1−/− mice were deeply anesthetized by intraperitoneal injection of a solution containing ketamine (10 mg/ml) and xylazine (1 mg/ml) in order to preserve muscle perfusion during dissection of both soleus and EDL muscles. Depth of anesthesia was assessed by the abolition of eyelid and pedal reflexes.

After dissection, the animals were killed by rapid neck dislocation. This protocol has been approved by the Animal Ethics Committee of the Catholic University of Louvain.

Soleus and EDL muscles were bathed in a 1-ml horizontal chamber continuously superfused with HEPES-buffered Krebs solution (100% O2) containing (in mM) 135.5 NaCl, 5.9 KCl, 1.0 MgCl2, 2.5 CaCl2, 1.6 HEPES sodium, and 11.5 glucose, maintained at a temperature of 20 ± 0.1°C. One end of the muscle was tied to an isometric force transducer and the other end to an electromagnetic motor and length transducer (52). Stimulation was delivered through platinum electrodes running parallel to the muscles. Resting muscle length (L0) was carefully adjusted for maximal isometric force with 100-ms (EDL) or 300-ms (soleus) maximally fused tetani. Force was recorded on a high-speed pen recorder (Sanborn model 320) and digitalized at a sampling rate of 1 KHz with a PCI 6023E i/o card (National Instru-

Table 1. Sequences of oligonucleotide primers

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<th>Gene</th>
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</table>

TRPC, canonical transient receptor potential; Fwd, forward; Rev, reverse.
Muscle Protein Extraction and allowed to adhere to the bottom of the dish culture dishes coated with extracellular matrix basement membrane suspended in F-12/DMEM supplemented with 2% fetal bovine serum were removed from this solution, washed twice in Krebs buffer, and the Catholic University of Louvain. Flexor digitorum brevis (FDB) Cantonal Veterinary Office and by the Animal Ethics Committee of Swiss Federal Law on Animal Welfare, and were approved by the with the Swiss Federal Veterinary Office’s guidelines, based on the cycle) over 2 min.

To investigate muscle fatigue, soleus muscles were subjected to 12- to sixteen week-old mice were killed by cervical dislocation. All procedures involving animals were performed in compliance with the Swiss Federal Veterinary Office’s guidelines, based on the Swiss Federal Law on Animal Welfare, and were approved by the Catholic University of Louvain. Flexor digitorum brevis (FDB) muscles were removed and incubated for 36 min at 37°C in Krebs solution containing 0.2% collagenase type IV (Sigma). The muscles were removed from this solution, washed twice in Krebs buffer, and suspended in F-12/DMEM supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin (Sigma). Single fibers were mechanically dissociated by passing the muscle repeatedly through fire-polished Pasteur pipettes. Dissociated fibers were plated onto tissue culture dishes coated with extracellular matrix basement membrane (Harbor Bio-products) and allowed to adhere to the bottom of the dish for 2 h.

Muscle Protein Extraction

Muscle protein extraction was done as previously described (64). Briefly, whole EDL muscle was homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 500 μl of ice-cold lysis buffer containing 50 mM Tris · HCl (pH 7.5), 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Homogenates were centrifuged 10 min at 10,000 rpm (Sorvall SS-34 rotor) to pellet myofibrillar proteins. These were resuspended in 9 M urea-50 mM Tris · HCl, pH 7.5. The supernatant contained soluble proteins. Myofibrillar and soluble muscle protein contents were determined by using the Bradford protein assay (Bio-Rad, Munich, Germany).

Myosin Heavy Chain Isoform Distribution

Myosin heavy chain (MHC) isoforms were separated as described previously (56) with only slight modifications. Muscles were frozen in liquid nitrogen, ground to powder, and homogenized in SDS solution containing (in mM) 40 dithiothreitol, 5 EDTA, and 100 Tris · HCl (pH 6.7), 4 mM EDTA, 0.4% (wt/vol) SDS, 0.1% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel consisted of 30% (vol/vol) glycercol, 8% (wt/vol) acrylamide-N,N'-methylenebisacrylamide (Bis; 99:1), 0.2 M Tris · HCl (pH 8.8), 0.1 M glycine, 0.4% (wt/vol) SDS, 0.1% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) N,N,N’,N’-tetramethylethylenediamine (TEMED). The stacking gel consisted of 30% (vol/vol) glycercol, 4% (wt/vol) acrylamide-Bis (50:1), 70 mM Tris · HCl (pH 6.7), 4 mM EDTA, 0.4% (wt/vol) SDS, 0.1% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) TEMED. The lower running buffer consisted of 0.05 M Tris (base), 75 mM glycine, and 0.05% (wt/vol) SDS. The upper running buffer was at six times the concentration of the lower buffer, but 0.12% (vol/vol) 2-mercaptoethanol was added. Electrophoresis was performed at a constant voltage of 60 V for 22 h, except for the first 40 min (stirring gel penetration), when the current was limited to 10 mA. Bands were scanned and quantified by densitometry.

Histology

Three- and twelve week-old mice were chosen for histological investigations. Both soleus and EDL muscles were dissected, fixed in 10% formalin-PBS on ice for 4 h, embedded in paraffin, and sectioned. Sections were deparaffinized with xylol, rehydrated with alcohol, fixed in 1% acetic acid in ethanol, and air dried. After incubation for 3 min with acidic hemalum, plates were rinsed, treated for 30 s with saturated lithium carbonate, rinsed, and counterstained with 1% eosin. After dehydration with ethanol and xylol, sections were mounted with Entellan (Merck). The size of muscle fiber sections was measured with a homemade planimetry program.

Patch-Clamp Recordings

Cell-attached patch-clamp recordings were performed at room temperature on individual fibers isolated as described above. Fibers were pretreated with 30 μM N benzyl-p-toluene sulfonamide (BTS) for 10 min to inhibit contractions (18) and immersed in a high-K+ solution composed of (in mM) 10 NaCl, 142 KCl, 2 MgCl2, 0.2 CaCl2, 5 glucose, 0.5 EGTA, and 10 HEPES (pH 7.3). Cells immersed in this solution had resting membrane potentials of 0 ± 2 mV, as verified by measuring membrane potential with sharp electrodes as described previously (n = 6) (40). Patch pipettes contained (in mM) 110 CaCl2, 10 HEPES (pH 7.3) supplemented with 2 mM tetraethylammonium (TEA), 20 μM 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS), and 1 μM nifedipine to inhibit K+ Cl−, and L-type Ca2+ currents. When needed, 100 μM 2-aminoethoxydiphenyl borate (2-APB; Alexis, Lausen, Switzerland) was added to the solution. Pipettes were pulled with a horizontal puller P2000 (Sutter Instrument, Novato, CA) and had a resistance of 3–5 MΩ. Electric currents were acquired and low-pass filtered at 2 kHz with a Multiclamp 700B amplifier and then digitized with a Digidata 1322 digitizer (Molecular Devices, Sunnyvale, CA) at 10 kHz.

Electrical properties of the channels were studied by measuring activity at series of potentials from −60 to +40 mV in steps of 20 mV, and single-channel currents at these potentials were measured by producing all-point histograms in the regions of TRPC1 activity and performing Gaussian fits to the established peaks. Store-dependent Ca2+ entry was studied by measuring currents at the holding potential of −40 mV; first basal activity was acquired for 5 min, then bath solution was replaced with equivalent high-K+ solution containing 1 μM thapsigargin, and the data acquisition was continued for an additional 20 min. Acquired currents were analyzed with the help of the single-channel search algorithm of the Clampex-10 program with conductance levels set to correspond to typical single-channel currents of TRPC1 or higher-conductance channel observed in patches (typically 0.7 or 2 pA corresponding to the holding potential of −40 mV), yielding channel activity (Np) values that were then used to calculate average currents corresponding to a particular mode of Ca2+ entry. Mechanosensitive properties of TRPC1 channels were studied in cell-attached patches held at −40 mV with the suction protocol consisting of steps to suction levels varying from 0 to −100 mmHg in steps of 10 mmHg, applied for 0.5 s, followed by a period of relaxation of 2.5 s, with an HSPC-1 instrument (ALA Scientific Instruments, Westbury, NY) and analyzed similarly to store-dependent currents.

Measurements of [Ca2+], and Influx of Ca2+

Muscle fibers were loaded for 1 h at room temperature with the membrane-permeant Ca2+ indicator fura-PE3/AM (1 μM) and Pluronic F-127 (0.004%). Before electrical stimulation, they were incubated in the presence of BTS (30 μM) to prevent contraction (18). Cells were illuminated through an inverted Nikon microscope (×40 magnification objective) alternatively at 340 and 380 nm, and the fluorescent light emitted at 510 nm was measured with a Deltascan spectrophluorimeter (Photon Technology International). The ratio R340/380 of the fluorescence intensity emitted at the two excitation wavelengths was calculated, and [Ca2+]i was determined with a previously described calibration (71).

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To measure the influx of Ca\(^{2+}\) into muscle fibers, 1 mM MnCl\(_2\) was added to the Krebs medium and the influx of Mn\(^{2+}\) through Ca\(^{2+}\) channels was evaluated by the quenching of fura-PE3 fluorescence excited at 360 nm, i.e., at the isosbestic point. The rate of quenching reflected the rate of Ca\(^{2+}\) entry (33, 55). Simultaneously, the ratio of fluorescence obtained with 340-nm and 360-nm excitation wavelengths (F\(_{340/360}\)) evaluated [Ca\(^{2+}\)]. Mn\(^{2+}\) entry was first measured at rest and then after Ca\(^{2+}\) was released from the stores with 20 mM caffeine and 1 μM thapsigargin (14).

In Vivo Experiments

Running wheel. Each mouse was individually housed in a standard mouse cage containing a low-inertia running wheel. Wheel-running distances were measured during the active part of the day (from 6 PM to 6 AM).

Escape test. For these experiments, the method initially devised by Carlson and Makiejus (16) was applied. Briefly, 4-mo-old mice were placed in front of a tube, and a cuff was wrapped around the tail and connected to a fixed force transducer. In response to gentle pinching of the tail, the mice tried to escape into the tube; this was prevented by the attachment of the tail to the force transducer, and a short peak of force was recorded. The procedure was repeated at short intervals over 2.5 min. The mean of the five highest peaks recorded was calculated for each animal and divided by its body weight (“total body force,” mN/g).

Forced treadmill exercise. The mice were placed on a homemade treadmill to run with an uphill inclination of 30° at a speed of 5 m/min for 5 min, followed by a progressive increase in speed of 1 m/min every minute up to 17 m/min. The back of the treadmill was equipped with a grid that discharged a mild electrical current, a stimulus aimed at motivating the animal to keep running on the treadmill. The test was stopped when the mouse remained on the shocker plate for 20 s without attempting to reengage the treadmill, and the time to exhaustion was determined.

Wire test protocol. In this test, animals were suspended by their forelimbs from a 1.5-mm-thick, 60-cm-long metallic wire at 45 cm above soft ground (59). The time until the mouse completely released its grasp and fell down was recorded. Three trials were performed per session, with a 30-s recovery period between trials. The maximum time per trial was set to 180 s. For each mouse, the scores of the three trials were averaged.

Statistics

Data are presented as means ± SE. ANOVA and Student’s t-tests and χ\(^2\), Pearson’s test were used to determine statistical significance.

RESULTS

Expression of TRPC1 and Ca\(^{2+}\) Fluxes in Muscle Fibers

We previously reported (49) that in myoblasts TRPC1 is by far the most widely expressed channel of the TRPC subfamily (at least 100 times more than other isoforms). In adult muscle fibers, we observed that TRPC1, TRPC2, and TRPC3 were the most abundantly expressed TRPC isoforms. It is important to note that in soleus muscle TRPC1 was the most expressed TRPC isoform, whereas this expression was a little bit weaker in EDL and tibialis anterior, where TRPC3 was the predominant isoform. As shown previously, TRPC5 and TRPC7 isoforms are less abundantly expressed (Fig. 1A) (71).

In several investigations, TRPC1 has been reported to be responsible for store-operated Ca\(^{2+}\) entry. In a recent study, we indeed confirmed that repression of TRPC1 in C2C12 myoblasts reduced the store-operated Ca\(^{2+}\) entry (49). Here, we investigated the role of TRPC1 by using TRPC1 knockout mice. The abolition of TRPC1 expression was confirmed by PCR, which showed the absence of TRPC1 mRNA in skeletal muscles of TRPC1 knockout mice. We found that in these muscles TRPC isoforms other than TRPC1 presented an ex-
pression pattern that was similar to that observed in muscles from TRPC1+/+ mice (Fig. 1B). To study the role of TRPC1 on Ca2+ fluxes, we evaluated the entry of Ca2+ by microspectrofluorometry and measured Ca2+ currents by the patch-clamp technique in muscle fibers isolated from FDB muscles.

Ca2+ influx was monitored by the Mn2+ quenching of fura-PE3 fluorescence. Mn2+ entry was first measured at rest, and then the release of Ca2+ from the reticulum was triggered by stimulation with 20 mM caffeine and 1 μM thapsigargin (used to activate ryanodine receptors and to inhibit SERCA pumps, respectively) (Fig. 2, A and B). We observed that the rate of quenching of fura-PE3 was similar in muscle fibers from TRPC1+/+ and TRPC1−/− mice at rest. Depletion of the stores by stimulation with thapsigargin significantly increased the quenching rate in both TRPC1+/+ and TRPC1−/− fibers (2-way ANOVA, \( P < 0.05 \)), but no difference was observed between TRPC1+/+ and TRPC1−/− fibers (Fig. 2C), suggesting that TRPC1 is not indispensable for store-operated entry of Ca2+ and does not play a major role in the basal influx of Ca2+ (at least through Mn2+-permeable Ca2+ channels). In addition, we verified that FDB muscles from TRPC1−/− mice did not change their expression of Stim1 and Orai1, two well-known proteins involved in store-operated influx of Ca2+ (data not shown).

For patch-clamp experiments, muscle fibers were immersed in the relaxing Ca2+-free solution utilized to emphasize currents through store-operated channels (57), and currents were recorded in cell-attached configuration at a series of potentials from −60 to +40 mV in steps of 20 mV (Fig. 3). To investigate store-operated responses, activity was recorded for 5 min in cell-attached patches held at a constant potential of −40 mV. Fibers were then stimulated with 1 μM thapsigargin, and channel activity was recorded over an additional 20 min (Fig. 3C). Typically, patches exhibited Ca2+ currents through essentially two ion channel types, one of small conductance and one of large conductance. Figure 3A shows an example of the small-conductance channel activity recorded at the series of potentials, as indicated on the right. The current–voltage relationship showed a slope conductance of \( 13 \pm 1 \text{ pS} \) \((n = 7)\) (Fig. 3B), in agreement with previous reports of TRPC1 activity in overexpression systems (9, 48, 63). Such activity was unambiguously observed in 19% of patches from TRPC1+/+ fibers (11 of 52 patches), an occurrence frequency similar to that observed in wild-type muscle fibers (6 of 31 patches from C57BL/6J mice). However, it was completely absent in TRPC1−/− muscle fibers (0 of 45 patches), allowing us to identify TRPC1 as the channel responsible for this small conductance. As expected (62), it was also significantly inhibited in the presence of 100 μM 2-APB (occurrence falling down to 0%; \( P < 0.05 \), χ²-test). We observed that the channel activity did not significantly increase after thapsigargin stimulation (mean current, in traces where activity was observed, measured at −40 mV over a period of 4 min, of 15 ± 8 fA before and 18 ± 11 fA after thapsigargin stimulation, \( n = 7 \), paired Student’s \( t \)-test comparing values.

Fig. 3. Ca2+ currents in cell-attached patches. 
A. A series of traces showing typical single-channel currents in TRPC1+/+ cells in cell-attached configuration at indicated voltages that were not observed in TRPC1−/− cells. B. Current–voltage relationship of TRPC1 channel \((n = 7)\). C. Sample trace showing Ca2+ current evoked in response to sarcoplasmic reticulum (SR) emptying by thapsigargin (Tg). Patch was held at −40 mV and first basal activity was acquired, followed by the application of thapsigargin-containing solution, as indicated by vertical arrow.
before and after stimulation yields $P = 0.85$; when normalized per all acquired traces mean currents become $3.2 \pm 1.5$ fA before and $3.8 \pm 2$ fA after thapsigargin, clearly showing that TRPC1 activity is not store dependent. In all observed cases, TRPC1 gating was happening in bursts. Unfortunately, the low frequency of observation of such activity (only 3 of 11 patches contained multiple bursts of TRPC1 activity) allowed us to put only a lower boundary estimate on interburst interval at $590 \pm 100$ s with a mean burst duration of $29.5 \pm 10$ s and in-burst $NP_0$ of $0.48 \pm 0.08$.

To assess mechanosensitive properties of the TRPC1 channel we performed measurements of $\text{Ca}^{2+}$ currents in cell-attached patches stimulated by application of suction ranging from 0 (no suction) to $-100$ (stress level often causing patch membrane rupture) mmHg in steps of 10 mmHg. Activity of stretch-activated channels (SAC) could often be evoked with such stimulation (see below); however, application of suction did not cause appearance of additional TRPC1 activity. In 2 cases out of 31 experiments, application of suction occurred when a burst of TRPC1 activity was ongoing. In both cases, the activity stopped with application of suction without reappearing at higher suctions (data not shown). These observations lead us to conclude that TRPC1 channel is not activated by mechanical stimulation.

Membrane patches of TRPC1$^{+/+}$ fibers also exhibited currents of larger amplitude, corresponding to a conductance of $>50$ pS and activity increasing on suction application. These currents could thus be associated with the activity of SAC with properties similar to those described in the literature (61, 68). In our experimental conditions (holding potential of $-40$ mV and $110$ mM CaCl$_2$ in the pipette), this conductance was observed in $\sim70\%$ of patches (37 of 52 patches) and was responsible for a mean overall current of $23 \pm 6$ fA (empty traces included). From mean current and occurrence values, we

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Fig. 4. Loss of TRPC1 induces a reduction of force production. A: maximal stress (force per cross-sectional area) developed during a 300-ms tetanus stimulated maximally (125 Hz). *$P < 0.05$ vs. TRPC1$^{+/+}$; **$P < 0.01$ vs. TRPC1$^{+/+}$ (Student’s $t$-test, n = 9). B and C: stress vs. stimulation frequency relationship in soleus muscles. *$P < 0.05$ vs. TRPC1$^{+/+}$ (Student’s $t$-test, n = 9). D: SDS-PAGE analysis of myosin heavy chain (MHC) isoforms of TA, soleus, and EDL muscles from TRPC1$^{+/+}$ (+) and TRPC1$^{-/-}$ (-) mice.
calculated that this conductance therefore seems responsible for ~88% of the entry of Ca\(^{2+}\) in muscle fibers at rest, the other 12% being due to TRPC1. The activity of this channel increased about twofold after thapsigargin-induced depletion of Ca\(^{2+}\) stores, to a mean current of 40 ± 10 fA (n = 28, P = 0.06). In TRPC1\(^{--}\) fibers, this conductance was observed with a somewhat smaller occurrence (27 of 45 patches). The related mean current tended to be smaller than that observed in TRPC1\(^{+/+}\) fibers, both at rest (14 ± 6 fA, n = 23) and after thapsigargin stimulation (29 ± 11 fA, n = 23; P = 0.19), although the difference did not reach statistical significance (comparing activity in TRPC1\(^{+/+}\) and TRPC1\(^{--}\) cells before and after thapsigargin application yields P = 0.31 and 0.47, respectively).

**Morphology, Phenotype, and Force Production of Muscles of TRPC1\(^{--}\) Mice**

To evaluate muscle function and structure, we carried out mechanical and histological experiments. Isolated soleus and EDL muscles were maximally stimulated to obtain fused tetani. Muscles from TRPC1\(^{--}\) mice had a strictly normal weight but developed a significantly reduced maximal tetanic force and stress compared with their controls (Fig. 4A and Table 2). Twitch parameters [time to peak (TTP) and half-relaxation time (1/2RT)] were not different between TRPC1\(^{+/+}\) and TRPC1\(^{--}\) muscles (Table 2). Investigations of force-frequency relationships in soleus and EDL muscles highlighted the lower stress produced by TRPC1\(^{--}\) muscles (Fig. 4B), but we could not detect any significant shift in sensitivity to the frequency of stimulation in soleus muscle (Fig. 4C) or in EDL (data not shown), suggesting that the muscles did not change their fast or slow phenotype.

This was further examined by analyzing MHC isoforms in tibialis anterior, soleus, and EDL muscles (Fig. 4D). Proteins corresponding to MHC isoforms were separated by SDS-PAGE in glycerol-containing gels. We did not observe any significant change in isoform distribution between muscles from TRPC1\(^{+/+}\) vs TRPC1\(^{--}\) mice. Indeed, soleus muscles from TRPC1\(^{+/+}\) and TRPC1\(^{--}\) mice contained 17% and 15% of MHC2X, the rest being MHC2B, and EDL muscles contained 20% and 18% of MHC2X, the rest being MHC2B (n = 4–5). Tibialis anterior muscles contained 26% and 24% of MHC2X, the rest being MHC2B, and EDL muscles contained 12% and 11% of MHC2X, the rest being MHC2B (n = 3–5). Trace amounts of MHC2A were detected in tibialis anterior and EDL muscles from both TRPC1\(^{+/+}\) and TRPC1\(^{--}\) mice, but their levels were too low to be quantified, while no trace of MHC1A was found in EDL muscle.

Comparative analysis of histological sections of muscles from 3-mo-old TRPC1\(^{+/+}\) and TRPC1\(^{--}\) mice did not reveal any major differences (Fig. 5, A–D). In particular, we did not observe any sign of myopathy such as necrosis, central nucleation, or fibrosis. Measurements of the cross-sectional area of the fibers were performed by planimetry. At least 100 fibers were measured in each EDL and 200 fibers in each soleus muscle, in order to determine the mean cross-sectional area of the fibers in a particular muscle. This was repeated on seven different animals and revealed an important decrease of fiber size in both EDL and soleus muscles from TRPC1\(^{--}\) mice compared with their control littermates [EDL 1,195.7 ± 72 and 939.9 ± 42 μm\(^2\) (P < 0.05 n = 7) and soleus 985.7 ± 38 and 859.9 ± 28 μm\(^2\) (P < 0.05, n = 7) in TRPC1\(^{+/+}\) and TRPC1\(^{--}\) muscles, respectively]. Distribution patterns of cross-sectional areas of fibers from different animals were similar for a particular muscle type (Fig. 5B) but clearly showed a shift of the curve of cross-sectional area to smaller values in TRPC1\(^{--}\) muscles (P < 0.01, \(\chi^2\), Pearson’s test).

Finally, we measured the contents of myofibrillar proteins in EDL muscles from TRPC1\(^{+/+}\) and TRPC1\(^{--}\) mice and observed a significant reduction in TRPC1\(^{--}\) muscles [95.77 ± 4.28 μg/mg muscle in TRPC1\(^{+/+}\) (n = 9) vs. 72.67 ± 3.01 μg/mg muscle in TRPC1\(^{--}\) (n = 7); Student’s t-test, P < 0.01].

In conclusion, TRPC1\(^{--}\) muscles produce not only less total force but also less force per cross-sectional area, and their fibers are smaller and contain less myofibrillar proteins.

**Muscle Fatigue in TRPC1\(^{--}\) Mice**

We next investigated the role of TRPC1 in muscle fatigue. Soleus muscles were first chosen because of their dependence on oxidative metabolism, thus limiting the role of anaerobic processes in ionic changes and their contribution to muscle fatigue. In these muscles, TRPC1 was the most abundant TRPC isoform. These muscles were subjected to 50-Hz stimulation trains of 500-ms duration at 1-s intervals (50% duty cycle). Under control conditions, maximal tetanic force progressively declined and after 20–30 s relaxation became incomplete during the 0.5 s separating two successive stimulation periods (Fig. 6A). We previously showed the role of Ca\(^{2+}\) entry in this process. Indeed, in the absence of extracellular

Table 2. Muscle mechanics

<table>
<thead>
<tr>
<th></th>
<th>TRPC1(^{+/+})</th>
<th>TRPC1(^{--})</th>
<th>TRPC1(^{+/+})</th>
<th>TRPC1(^{--})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>28.09 ± 1.31</td>
<td>29.67 ± 0.99</td>
<td>28.64 ± 0.87</td>
<td>29.09 ± 1.58</td>
</tr>
<tr>
<td>Muscle mass, mg</td>
<td>8.95 ± 0.18</td>
<td>8.93 ± 0.08</td>
<td>10.64 ± 0.22</td>
<td>10.85 ± 0.39</td>
</tr>
<tr>
<td>L(_{0}), mm</td>
<td>12.28 ± 0.12</td>
<td>12.04 ± 0.23</td>
<td>11.76 ± 0.17</td>
<td>11.65 ± 0.16</td>
</tr>
<tr>
<td>TTP, ms</td>
<td>19.11 ± 0.80</td>
<td>20.56 ± 0.60</td>
<td>35.25 ± 1.73</td>
<td>34.38 ± 2.41</td>
</tr>
<tr>
<td>1/2RT, ms</td>
<td>31.78 ± 3.24</td>
<td>32.78 ± 2.97</td>
<td>59.17 ± 5.56</td>
<td>60.31 ± 5.33</td>
</tr>
<tr>
<td>F(_0), mN</td>
<td>374.13 ± 16.35</td>
<td>310.11 ± 18.21(^*)</td>
<td>181.28 ± 6.12</td>
<td>150.74 ± 8.69(^*)</td>
</tr>
<tr>
<td>S(_0), mN/mm(^2)</td>
<td>256.77 ± 13.05</td>
<td>209.10 ± 13.32(^*)</td>
<td>201.69 ± 7.82</td>
<td>163.17 ± 9.70(^*)</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>14</td>
<td>22</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are means ± SE. EDL, extensor digitorum longus; L\(_{0}\), resting muscle length; TTP, time to peak; 1/2RT, half-relaxation time; F\(_{0}\), maximal force; S\(_{0}\), maximal stress (force/cross-sectional area). \(^*P < 0.05\).
Ca\(^{2+}\), the decline of maximal tetanic force was faster and the relaxation during the 0.5-s period separating two successive stimulations was complete (24), suggesting that the latter might be due to cytosolic Ca\(^{2+}\) accumulation (3). Here, using the same protocol, we compared the time course of muscle fatigue in soleus muscles from TRPC1\(^{-/-}\) (A and C) and TRPC1\(^{-/-}\) (B and D) mice. E and F: fiber area distribution in EDL (E) and soleus (F) muscles from TRPC1\(^{+/+}\) and TRPC1\(^{-/-}\) mice. The distributions observed in TRPC1\(^{+/+}\) and TRPC1\(^{-/-}\) were significantly different (P < 0.01, \(\chi^2\), Pearson’s test).

We next studied muscle fatigue of EDL, a fast glycolytic muscle. The protocol was adapted to avoid the possibility of metabolic changes masking the possible role of Ca\(^{2+}\) entry in the process. Muscles were subjected to 125-Hz stimulation trains of 100-ms duration at 2-s intervals. We observed that maximal force declined faster in muscles from TRPC1\(^{-/-}\) than TRPC1\(^{+/+}\), similarly as described for soleus muscles (Fig. 6D).

To further investigate the role of Ca\(^{2+}\) entry and Ca\(^{2+}\) accumulation in the evolution of force during the protocol of fatigue, we measured [Ca\(^{2+}\)]\(_i\) transients during fatigue experiments. For technical reasons we performed these measurements on isolated fibers from FDB muscles. FDB muscle contains IIA and IIX fibers and therefore presents an intermediate metabolism (oxidative and glycolytic); we previously showed (60) that its contraction is much slower than that of EDL. We therefore used the same protocol of stimulation as described for soleus. As shown in Fig. 7, A and B, the amplitude of [Ca\(^{2+}\)], transients induced by
repetitive stimulations was maintained throughout the fatigue protocol in TRPC1+/− muscle fibers, indicating that the important loss of force observed under control conditions (Fig. 6) is not due to a decrease of Ca2+ release from the SR. However, in TRPC1−/− muscle fibers, we observed a small and progressive decrease of the amplitude of [Ca2+]i transients, which could explain the accentuated decline of force observed in TRPC1−/− muscles. We also observed that Ca2+ progressively accumulated in fibers after repetitive stimulation (Fig. 7C). Indeed, [Ca2+]i measured at the end of the relaxation period (limited to 500 ms) was almost 2.5 times higher at the end of the fatigue protocol than at the beginning. Interestingly, this was not observed in muscle fibers from TRPC1−/− mice, in which [Ca2+]i, at the end of the relaxation period returned to baseline levels throughout the protocol. This fits with the complete relaxation observed in TRPC1−/− muscles (Fig. 6C) and suggests that the incomplete relaxation observed in TRPC1+/+ muscles is due to an accumulation of [Ca2+]i in the cytoplasm.

**TRPC1 Knockout Mice Present In Vivo Signs of Fatigue**

To study the role of TRPC1 in skeletal muscle functions, we used four functional tests designed to investigate in vivo the ability of the animal to perform voluntary exercise (running wheel test and escape test) or endurance and forced exercise (treadmill test and wire test). The running wheel test was used to evaluate spontaneous voluntary exercise during the active part of the day. We did not observe any difference in the running distance between TRPC1+/+ and TRPC1−/− mice (Fig. 8B). Likewise, the escape test used to evaluate the whole body force developed by a mouse to escape in response to a stimulus (pinching of the tail) showed similar performances in TRPC1+/+ and TRPC1−/− mice (~200 mN/g) (Fig. 8A).
In contrast, TRPC1+/+ and TRPC1−/− mice performed very differently in the two endurance tests. These two tests were designed to evaluate the resistance of the animal to endurance exercise. As described in MATERIALS AND METHODS, treadmill exercise was performed in uphill inclination (30°). During the first 5 min mice were forced to run at 5 m/min, and then the speed was increased by 1 m/min up to 17 m/min. TRPC1+/+ mice ran easily during the progressive increase of treadmill speed and resisted beyond 45 min of the test. Interestingly, TRPC1−/− mice presented difficulties when speed was progressively increased and ran only <20 min (n = 10). The performance of the TRPC1−/− mice in the treadmill test was two to three times lower than that observed in a control population (P < 0.001, Fig. 8C), suggesting a predisposition to muscle fatigue. A similar result was obtained with the wire test, which also evaluates muscle force and resistance to fatigue. In this test, a mouse is suspended from a horizontal wire and the time until the mouse releases its grasp and falls down is recorded (test stopped after 180 s, 3 successive trials). We observed that TRPC1+/+ mice rarely fell down before the end of each trial. However, TRPC1−/− mice were unable to resist so long [57 ± 13 s (n = 11) in TRPC1−/− vs. 174 ± 4 s (n = 10) in TRPC1+/+ mice; Fig. 8D].

DISCUSSION

In this paper, we show that the influx of Ca2+ through TRPC1 channels represents a minor part of the entry of Ca2+ into muscle fibers at rest (≈12% of the total influx of Ca2+). We also clearly show that the activity of TRPC1 channels is not store dependent. However, TRPC1-related Ca2+ entry is detectable in muscle fatigue experiments. Indeed, TRPC1 channels modulate the entry of Ca2+ during repeated muscle contractions and allow muscle to maintain force production during sustained stimulations. Finally, we show that muscles from TRPC1−/− mice display a smaller fiber cross-sectional area and generate less force than their controls and express less myofibrillar proteins.

Patch-clamp experiments performed on adult fibers from TRPC1+/+ mice revealed the presence of two ion channel types, one of large conductance exhibiting mechanosensitive properties and characteristics similar to those described in the literature (24, 61, 68) and one of small conductance (13 pS). The small-conductance activity presented the characteristics of TRPC1 measured in overexpression systems (9, 48, 63), was inhibited by 2-APB, and was completely absent in TRPC1−/− fibers, strongly suggesting that this current is carried by TRPC1 channels. We clearly show that in adult skeletal muscle fibers TRPC1 is not store dependent. Although we cannot rule out an effect of the lack of TRPC1 on the presence or the activity of other (possibly unknown) channels, we show by fura-PE3 quenching experiments that, even if TRPC1 channels were present in the T tubules (where they cannot be patched), they do not play an indispensable role in store-operated entry of Ca2+. These results support previous observations performed in smooth muscle (22) but appear contradictory to the situation observed in myoblasts. Indeed, we previously showed (49) that in myoblasts a partial repression of TRPC1 [using a short hairpin RNA (shRNA) strategy] decreased the store-operated entry of Ca2+. Two observations can be proposed to explain this discrepancy. First, we showed that TRPC1 repres-
sion slowed down myoblast migration and differentiation. It has been shown that store-operated Ca\(^{2+}\)/H11001 entry increases during differentiation (67). It is therefore possible that the difference observed in the store-operated entry of Ca\(^{2+}\)/H11001 reflects the difference in myoblast-myotube maturation. Alternatively, this difference between myoblasts and adult muscle fibers could be due to the interaction of TRPC1 with myogenic factors. Indeed, TRPC1 has been shown to interact with the a-isoform of the inhibitor of myogenic family (I-mfa) (17, 51). I-mfa inhibits store-operated currents through the TRPC1 channel. However, the interaction between TRPC1 and I-mfa can be relieved by binding of myogenic factors such as myogenin to I-mfa. I-mfa has therefore been proposed to function as a molecular switch to suppress the store dependence of TRPC1. The binding of I-mfa to myogenic factors might explain our observations that TRPC1 seems to be store dependent in myoblasts (49) but not in adult fibers.

To investigate the role of TRPC1 in skeletal muscle function, we first compared the force produced by EDL and soleus muscles of TRPC1/H11002/H11002 and TRPC1/H11001/H11001 mice. We observed a significant decrease of force per cross-sectional area developed by muscles from TRPC1/H11002/H11002 mice, without any shift in the frequency-response curve. The decrease of force did not seem to be related to the maximal [Ca\(^{2+}\)]\(_{i}\) reached during contraction. Indeed, we did not observe a significant decrease of Ca\(^{2+}\) release in adult FDB fibers submitted to electrical stimulation (see Fig. 7A, 1st tetanus of experiment). Interestingly, similar results were obtained in Homer 1\(^{-/-}\) mice, in which the activity of TRPC1 channel seemed to be increased (67). In agreement with force measurements, we observed a decrease of 24% in the contents of myofibrillar proteins. We did not observe any major typical sign of myopathy such as necrosis, regeneration, central nuclei, or fibrosis. We did, however, observe a decrease of the mean cross-sectional area of fibers without any change in MHC isoform distribution (in particular, we did not observe any significant increase of type I fibers, which are smaller). This particular phenotype (smaller fibers with decreased content of myofibrillar proteins) might reflect a slower development that could be due, as mentioned above, to the interactions between TRPC1 and myogenic factors.

To unravel the possible role of TRPC1-related entry of Ca\(^{2+}\) in muscle function, we studied muscle fatigue in TRPC1/H11001/H11001 and TRPC1/H11002/H11002 mice. Muscle fatigue consists of a progressive decrease in relaxation speed and force production when muscles are stimulated maximally and repeatedly. It has been classically attributed to the accumulation of intracellular lactic acid (39) resulting in acidosis, but it is now clear that this effect contributes little to muscle fatigue (2) and that intracellular ionic changes play a major role in this process (2, 66). We hypothesized that the entry of Ca\(^{2+}\) might also modulate the amount of releasable Ca\(^{2+}\).
from the SR. For example, fast twitch muscles intermittently stimulated at 40 Hz for a total of 30 s show a net influx equivalent to 5–10% of the Ca\(^{2+}\) already present in the SR (3). Similarly, we previously showed (24) that in the absence of external Ca\(^{2+}\) a faster decline of force is observed. Here, we observed that the decline of force was faster in TRPC1\(^{-/-}\) than in TRPC1\(^{+/+}\) muscles. We therefore compared Ca\(^{2+}\) transients during all contractions of the fatigue protocol. In normal muscle, we did not observe any decrease of the [Ca\(^{2+}\)]i reached during successive tetani. This is probably due to the fact that, for technical reasons, all [Ca\(^{2+}\)]i measurements were performed in the presence of BTS to avoid movement artifacts, a situation that minimizes ATP consumption and avoids P\(_i\) accumulation (and thus accumulation of Ca\(^{2+}\) phosphate in the SR). In contrast, the release of Ca\(^{2+}\) progressively decreased in muscle fibers from TRPC1\(^{-/-}\) mice, emphasizing the need for Ca\(^{2+}\) entry through TRPC1-related channel to allow maintenance of a normal level of [Ca\(^{2+}\)] in the SR and a normal production of force during repeated stimulations.

Soleus muscle fatigue was accompanied by a slowing of relaxation evidenced by an incomplete loss of force at the end of the 0.5-s period of relaxation between two contractions (Fig. 6). The mechanisms inducing this slowing of relaxation are still not completely understood (3). Some investigators have shown that relaxation slowing might be due to a decrease of SERCA pump activity. However, this effect might be partially counteracted by a progressive decrease of myofibrillar Ca\(^{2+}\) sensitivity (72, 73). Other studies have proposed a progressive alteration of cross-bridge function (42). Finally, metabolic changes related to fatigue such as the progressive acidosis and the accumulation of ADP and P\(_i\) exert their effect on myosin and on SERCA pump and may therefore also contribute to slowing of relaxation (4, 20, 26). Our experiments clearly show that slowing of relaxation is related to an accumulation of cytosolic Ca\(^{2+}\). Indeed, in the absence of TRPC1 channel, the release of Ca\(^{2+}\) from the SR decreases progressively and the accumulation of cytosolic Ca\(^{2+}\) at the end of the relaxation period observed in normal conditions disappears. The consequence is a complete relaxation during the whole experimental protocol of fatigue.

Finally, we investigated whether this susceptibility to fatigue had some repercussions in vivo. Interestingly, we observed that TRPC1\(^{-/-}\) mice performed much worse than TRPC1\(^{+/+}\) mice in the wire test and in the forced treadmill test, two tests designed to evaluate the resistance of the animal to endurance exercise. In contrast, these mice performed totally normally in the running wheel test and in the escape test, two voluntary exercises designed to grossly evaluate spontaneous activity and the force of the animal. We therefore propose that the loss of endurance observed in TRPC1\(^{-/-}\) mice is due, at least partially, to the diminution of force production observed in vitro as well as to the accentuation of muscle fatigue. Obviously, this does not exclude possible additional effects not related to muscle fatigue.

In conclusion, our results emphasize the importance of TRPC1-related entry of Ca\(^{2+}\) in maintaining force production during sustained trains of contractions and the importance of TRPC1 in the acquisition of adult muscle phenotype.

The gating mechanisms of TRPC1 are, however, still unclear. It might be constitutively active, and because it is responsible only for a small part of the total entry of Ca\(^{2+}\) at rest, its repression could be undetectable in FURA-PE3 quenching studies but emphasized under conditions where Ca\(^{2+}\) accumulates in the cytosol, as in fatigue experiments. Alternatively, TRPC1 channel might also be activated during contraction. However, we show that it is not store dependent. Launikonis and colleagues (46) recently described an excitation-induced Ca\(^{2+}\) current from the transverse tubular membrane; this current activates following single action potentials, but its biophysical properties plead against TRPC1. Finally, the TRPC1 isoform has been proposed to form a stretch-activated cation channel when artificially reconstituted in CHO cells (53). However, this issue is still controversial because, under physiological conditions, mechanosensitive gating has not been reported for this channel (22, 37). Here, we show that in the native environment of skeletal muscle fibers, the TRPC1 channel does not exhibit mechanosensitive properties.

In a previous study, we reported (71) that some TRP channel isoforms had an abnormally increased activity in dystrophin-deficient muscle fibers. The channel involved seemed to be store dependent and mechanosensitive but was insensitive to 2-APB (24, 29). The results presented here therefore suggest that the TRP channel detected in these dystrophic fibers was not TRPC1. In addition, another isoform (TRPV2) was recently implicated in the physiopathology of the disease (41, 76).

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


