

Functional role of store-operated and stretch-activated channels in murine adult skeletal muscle fibres

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In skeletal muscle, Ca^{2+} is implicated in contraction, and in regulation of gene expression. An alteration of $[\text{Ca}^{2+}]_i$ homeostasis is responsible, at least partially, for the muscle degeneration that occurs after eccentric contractions in Duchenne muscular dystrophy, a disease characterized by the loss of the cytoskeletal protein dystrophin. Using patch clamp in the cell-attached configuration, we characterized the store-operated channels (SOCs) and the stretch-activated channels (SACs) present in isolated mouse skeletal muscle. SOCs were voltage independent, had a unitary conductance between 7 and 8 pS (110 mM Ca^{2+} in the pipette), and their open probability increased when the sarcoplasmic reticulum was depleted by thapsigargin. These SOCs were identical to those previously described in the pathophysiology of Duchenne muscular dystrophy. Under the same experimental conditions, we detected a channel activity that was increased by applying a negative pressure to the patch electrode. The SACs responsible for this current had the same unitary conductance and current–voltage relationship as those observed for SOCs. SOCs and SACs had a similar sensitivity to pharmacological agents such as Gd^{3+} , SKF-96365, 2-aminoethoxydiphenyl borate and GsMTx4 toxin. Moreover, stimulation with IGF-1 increased the occurrence of the activity of both channel types. Together, these observations suggest that SOCs and SACs might belong to the same population or share common constituents. From a functional point of view, treatment of soleus muscle with SKF-96365 or GsMTx4 toxin increased its sensitivity to a fatigue protocol, suggesting that the influx of Ca^{2+} that occurs through these channels during contraction is also involved in force maintaining during repeated stimulations.

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In most cells, depletion of Ca^{2+} from intracellular Ca^{2+} stores causes Ca^{2+} entry across the plasma membrane. This process has been generally termed capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (Putney, 1986; Parekh & Penner, 1997), and is considered to play an important role in Ca^{2+} homeostasis. Candidate channels for this store-operated entry of Ca^{2+} are proteins of the transient receptor potential (TRP) superfamily, which were initially recognized in *Drosophila* (Montell & Rubin, 1989) and then also in mammalian cells (Birnbaumer *et al.* 1996; Okada *et al.* 1998; Putney, 1999). TRP proteins are cation channels presenting a similarity of structure (six transmembrane domains) and some sequence homology. In mammals, the TRP superfamily contains six subfamilies. Four of them share substantial sequence identity in the transmembrane domains: classical (TRPC), vanilloid (TRPV), melastatin (TRPM), and ANKTM1

(TRPA). The last two subgroups, i.e. muclopins (TRPML) and polycystins (TRPP) are only distantly related to other subfamilies. Studying the precise function of these channels has been rendered difficult because of the lack of specific inhibitors. Different drugs have been used such as trivalent lanthanides (La^{3+} and Gd^{3+}), and SKF-96365 (1- $[\beta$ -[3-(4-methoxyphenylpropoxy)]-4-methoxyphenetyl]-1*H*-imidazole) (Putney, 2001), but they lack specificity (Bales *et al.* 1999). Even 2-aminoethoxydiphenyl borate (2-APB), initially used as an inositol-trisphosphate-receptor blocker (Ma *et al.* 2000), has the ability to block thapsigargin-induced Ca^{2+} entry, which varies from cell to cell (Kukkonen *et al.* 2001; Ma *et al.* 2001), and obviously has multiple targets (Peppiatt *et al.* 2003).

So far, the TRP channels identified as possibly involved in store-operated influxes of Ca^{2+} belong to the TRPC and TRPV subfamilies. Indeed, using a methodology of heterologous expression, TRPC1, 2, 3, 4 and 5, and TRPV6, have been shown to be possibly activated by store depletion

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(Groschner *et al.* 1998; Philipp *et al.* 1998; Vannier *et al.* 1999; Warnat *et al.* 1999; Liu *et al.* 2000; Yue *et al.* 2001). However, other authors showed that heterologous expression of these proteins also gave channels whose activation was independent of store depletion (Zitt *et al.* 1997; Hofmann *et al.* 1999; Schaefer *et al.* 2000; Wu *et al.* 2000; Boddington *et al.* 2003). This might be due to the fact that the mechanism of channel activation depends on their expression level (Vazquez *et al.* 2003). Strategies using repression with antisense oligonucleotides were then used to demonstrate the possible involvement of TRPC1, 2 and 3, and TRPC4, in store-operated entry of Ca^{2+} (Philipp *et al.* 2000; Wu *et al.* 2000; Gailly & Colson-Van Schoor, 2001). Finally, the involvement of TRPC4 in store-operated Ca^{2+} current was demonstrated by showing that mice deficient in TRPC4 lack this current in endothelial cells and exhibit reduced vasorelaxation (Freichel *et al.* 2001).

In a previous work, we have shown that store-dependent channels are present in skeletal muscles and that their activity is abnormally increased in Duchenne muscular dystrophy (Vandebrouck *et al.* 2002), a myopathy due to the lack of a cytoskeletal protein called dystrophin.

TRP channels also respond to stimuli other than store depletion. Indeed, TRPC proteins have been shown to respond to agonists independently of store depletion, and TRPV proteins are sensitive to heat and cold, pH changes, osmolarity or volume changes, or are constitutively active and responsible for Ca^{2+} reabsorption in kidney and duodenum (TRPV5 and 6) (reviewed in Benham *et al.* 2002; Minke & Cook, 2002). In the present study, we characterize the pharmacological profile and the different modes of activation of voltage-independent Ca^{2+} channels present in adult skeletal muscle fibres, and we assess their possible involvement in the normal mechanical response of muscle to electrical stimulation.

Methods

Muscle mechanics

Adult wild-type (C57) mice were deeply anaesthetized with a solution (10 ml kg^{-1} , intraperitoneally) containing ketamine (10 mg ml^{-1}) and xylazine (1 mg ml^{-1}) in order to preserve muscle perfusion during dissection of both soleus muscles. Depth of anaesthesia 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, Brussels. Soleus muscles were bathed in a 1 ml horizontal chamber continuously superfused with oxygenated Krebs solution (O_2 95%/CO₂ 5%) containing (mM): NaCl 118, NaHCO₃ 25, KCl 5, KH₂PO₄ 1, CaCl₂ 2.5, MgSO₄ 1, glucose 5, maintained at a temperature of $20 \pm 0.1^\circ\text{C}$. One end of the muscle was tied to an isometric force transducer, and the other end to an electromagnetic motor and length

transducer. Stimulation was delivered through platinum electrodes running parallel to the muscles. Muscle length was carefully adjusted for maximal isometric force using 0.35 s maximally fused tetani. Force was recorded on a high-speed pen recorder (Sanborn model 320).

Isolation of adult skeletal muscle fibres

The flexor digitorum brevis (FDB) muscles were removed and incubated for 38 min at 37°C in an oxygenated 'Krebs-Hepes' solution (see composition below) containing 0.2% collagenase type IV (Sigma, St Louis, MO, USA). Muscles were then washed twice in Krebs buffer, suspended in DMEM/HAM F12 (Sigma) supplemented with 2% fetal bovine serum (Sigma), and mechanically dissociated by repeated passages through fire-polished Pasteur pipettes of progressively decreasing diameter. Dissociated fibres were plated onto tissue culture dishes coated with extracellular matrix basement membrane (Harbor Bio-Products, Norwood, MA, USA) and allowed to adhere to the bottom of the dish for 2 h. For Ca^{2+} measurements, cells were plated on circular glass coverslips. Culture dishes were kept in an incubator, with 5% CO₂ at 30°C .

Measurements of cytosolic [Ca^{2+}]

Muscle fibres were loaded for 1 h at room temperature with the membrane-permeant Ca^{2+} -indicator Fura-PE3/AM (1 μM) and Pluronic F-127 (0.004%). Fura-PE3/AM was preferred to Fura-2/AM as it is stable during long-lasting experiments, with little or no compartmentation (Vorndran *et al.* 1995). Fibres were illuminated through an inverted Nikon microscope ($\times 40$ -magnification objective) alternately at 340 and 380 nm, and the fluorescent light emitted at 510 nm was measured using a Deltascan spectrofluorimeter (Photon Technology International). The ratio $R_{340/380}$ of the fluorescence intensity emitted at the two excitation wavelengths was calculated, and cytosolic concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) was determined with a calibration previously described (Vandebrouck *et al.* 2002). The Krebs-Hepes solution contained (mM): NaCl 135.5, MgCl₂ 1.2, KCl 5.9, glucose 11.5, Hepes 11.5, CaCl₂ 1.8 (pH 7.3). When necessary, CaCl₂ was omitted and replaced by 50 μM sodium EGTA, and osmolarity was adjusted with sucrose. The potassium aspartate solution contained (mM): potassium aspartate 150, MgCl₂ 5, EGTA 10 and Hepes 10 (pH 7.3).

Electrophysiological methods

Single-channel activity was recorded from cell-attached patches using the technique described by Hamill *et al.* (1981). Patch electrodes were pulled on a DMZ-Universal (Zeitz-Instruments) puller in three stages

from borosilicate glass capillaries (1.5 mm in diameter; Harvard Apparatus) to a tip diameter of 1–2 μm . Patch electrodes had a resistance of 2–5 M Ω . Cells were viewed under phase contrast with a Diaphot Nikon inverted microscope. The activity was recorded at a constant holding potential of –60 mV and at room temperature, using a HEKA EPC-9 amplifier. This holding potential value takes into account the basal membrane potential (measured independently at –50 mV) (Cahalan & Neher, 1992). Current records were filtered with a Bessel filter at 3 kHz and digitized at 10 kHz. Data were analysed using Pulse-Fit, Pulse-Tools and Origin 6.1 software. Most of the patches contained more than one channel. Therefore, the global open probability ($n.P_o$) was calculated (ratio of total open time to total time for a given patch).

The intrapipette solution contained (mM): CaCl₂ 110, Hepes 10 and DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) 0.1. The bathing solution contained (mM): NaCl 124, MgCl₂ 1.2, KCl 5.9, glucose 11.5, HepesNa 11.5, EGTA 10. The osmolarity (measured with a micro-osmometer type 13/13DR Roebling) of these solutions was adjusted to 320–330 mosmol l⁻¹ by adding water or sucrose, and adjusted to pH 7.3 with NaOH. DIDS was used to block possible chloride conductances.

Thapsigargin (TG) was dissolved in DMSO and diluted 1:2000 into the bath to a final concentration of 1 μM . Channels activity was recorded during 90 s (30 sweeps of 3 s) in the absence of TG (control condition) and was prolonged for 3–5 min after application of the drug on the same patch. The solvent alone had no effect on channel activity. Mechanical stimulation was performed by applying on the patch pipette a suction the pressure of which was measured with a mercury-filled U-shape manometer. Channel mechanosensitivity was therefore only studied under stationary conditions. As the response to stretch was fast, channels activity was only recorded during 30–60 s for each chosen pressure (one or two series of 10 sweeps of 3 s).

Reagents

The GsMTx4 toxin, isolated from *Grammostola spatulata* spider (Suchyna *et al.* 2000), was obtained from Peptides International (Louisville, KY, USA); SKF-96365 and 2-APB were from Alexis Corporation (Lausen, Switzerland); and Fura-PE3/AM was from Calbiochem (Darmstadt, Germany). All other reagents were of analytical grade and purchased from Sigma. Channel inhibitors were added to the pipette solution in order to have access to the external face of the channel in cell-attached configuration.

Statistics

Data are presented as means \pm s.e.m.). The χ^2 test, Fisher exact test, ANOVA and Student's *t* test were used to determine statistical significance.

Results

Characterization of TG-induced Ca²⁺ currents in skeletal muscle fibres

In a previous paper we showed the presence, in muscle fibres, of voltage-independent Ca²⁺ channels that are activated by store depletion. Under the conditions used here (cell-attached configuration, muscle fibres in physiological medium), we detected a basal Ca²⁺ channel activity with a unitary conductance of 7.4 ± 0.47 pS ($n = 7$, in 110 mM Ca²⁺) and a reversal potential at +43 mV (Fig. 1A and B). TG was used to deplete the stores by inhibiting the sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA). After 90 s of patch recording under control conditions, 1 μM TG was added to the bath and recording was prolonged by 3 min. As previously shown (Vandebrouck *et al.* 2002), TG did not induce significant modifications of the properties of these channels (unitary conductance of 7.1 ± 0.68 pS, $n = 20$, reversal potential of +48 mV). However, their occurrence (number of patches in which a Ca²⁺ current is recorded/number of patches sampled) was significantly increased by a factor of 2.8 in the presence of TG (the channel activity appearing in patches devoid of activity in the absence of TG, Fig. 1C). The open channel probability (P_o) was also significantly increased after TG application (0.05 ± 0.02 ($n = 7$) versus 0.19 ± 0.08 ($n = 20$)) and the quantity of charge passing through the channels (integration of the current extrapolated over a period of 120 s of observation) approximately doubled (Fig. 1C). The current passing through these channels was efficiently inhibited by 50 μM Gd³⁺, 50 μM La³⁺ or 30 μM SKF-96365, but not by 100 μM 2-APB (Fig. 1A, D and E). A dose–response relationship is presented for Gd³⁺, showing an IC₅₀ value of around 30 μM (Fig. 1D). All these properties were consistent with previously described properties of voltage-independent Ca²⁺ channels found in skeletal muscle (Vandebrouck *et al.* 2002), and presented some similarities with the channels studied previously by Franco-Obregon and Lansman and reported to be mechanosensitive (Franco-Obregon & Lansman, 1994). This prompted us to investigate whether the store-operated channels detected here were also mechanosensitive.

Characterization of stretch-induced Ca²⁺ currents in skeletal muscle fibres

Figure 2 shows that applying a negative pressure to the patch electrode increased the P_o of voltage-independent Ca²⁺ channels but had no significant effect on their conductance (7.1 ± 0.39 pS, $n = 6$) or on their reversal potential (+52 mV), which were similar to those described above (Fig. 2A and B). The relationship between the amount of pressure applied to the patch electrode and

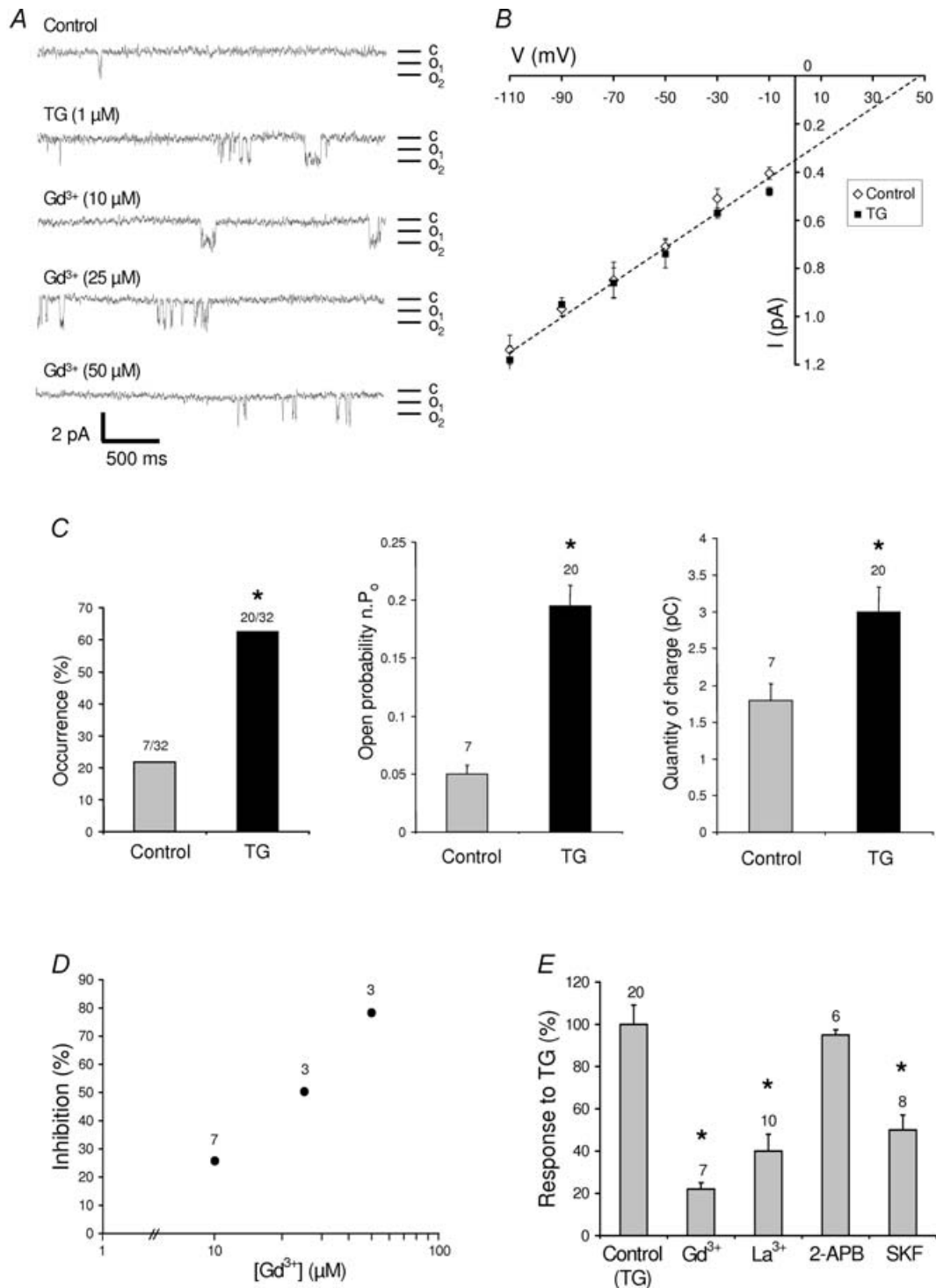


Figure 1. Characterization of thapsigargin-induced Ca^{2+} currents in skeletal muscle fibres

A, examples of current traces (patch-clamp; cell-attached configuration at -60 mV holding potential) recorded (in different patches) under control conditions, after stimulation with $1 \mu\text{M}$ thapsigargin (TG), or in the presence of TG and various concentrations of Gd^{3+} . Mean unitary currents between 0.65 and 0.73 pA. c, closed state; o, open states. *B*, current–voltage relationship in control (\diamond) and TG-treated cells (\blacksquare). Voltages indicated were determined from the applied holding potential and the estimated resting potential (-50 mV). *C*, the proportion of patches with a channel activity (occurrence), the open state probability ($n.P_o$), and the quantity of charge obtained by integration of the current observed extrapolated over a period of 120 s under control condition and after application of $1 \mu\text{M}$ TG (same patch studied). *Significant difference ($P < 0.05$) between control and TG-treated cells; the number of patches under each condition is indicated above the histograms. χ^2 test (for comparison of occurrences) and

channel P_o was well fitted by a Boltzmann equation (Fig. 2C). In contrast, the occurrence of channel activity was not significantly modified by mechanical stretch (Fig. 2D). We also compared the response to mechanical stretch in the presence and the absence of TG. Figure 2C shows that channel activity recorded from muscle fibres under the two experimental conditions increased with the pressure of suction applied to the patch electrode. The occurrence (Fig. 2D) and the total quantity of charge (not shown) also significantly increased. To further determine whether store-dependent channels and mechanosensitive channels belong to the same population, we studied the sensitivity of these channels to pharmacological agents. We found that inhibitors of store-operated channels, such as Gd^{3+} ($50 \mu M$, i.e. just above the EC_{50} value for SOC inhibition, see above) and SKF-96365 ($30 \mu M$) which inhibited, respectively, 78 and 50% of the current induced by TG (Fig. 1E) also inhibited 78 and 69%, respectively, of the current triggered by pressure application (Fig. 2F). We also tested GsMTx4, a peptide toxin from the tarantula *Grammostola spatulata*, reported to specifically block mechanosensitive channels (Suchyna *et al.* 2000). In the presence of $5 \mu M$ GsMTx4, no basal activity was detected. As expected, $5 \mu M$ GsMTx4 inhibited the response to stretch (suction from -20 to -60 mmHg), but interestingly, also completely abolished the response to TG (no activity detected in eight experiments, representing a total recording of more than 43 min in the presence of TG; Fig. 3). We concluded that adult muscle fibres have SOCs and SACs that share similar pharmacological profiles. In particular, both channels are inhibited by the GsMTx4 toxin which has been shown to inhibit TRPC1 channels.

Among the stretch-activated channels described in skeletal muscle, the growth-factor-regulated channel (GRC, now named TRPV2) has been shown to translocate to plasma membrane upon IGF-1 stimulation (Kanzaki *et al.* 1999; Iwata *et al.* 2003). Interestingly, we observed that the occurrence of channel activity (measured under basal conditions or after stimulation by -20 mmHg stretch or by $1 \mu M$ TG) increased when the cells were pretreated for 5 min with 2 nM IGF-1, suggesting that the channels detected here might also be constituted, at least partially, of TRPV2 (Fig. 4).

Physiological activation of store-operated channels

Experimentally, sarcoplasmic reticulum can be fully depleted by a combined and prolonged action of 20 mM caffeine and $1 \mu M$ TG in the absence of external Ca^{2+}

(10 mM EGTA). Re-introduction of Ca^{2+} in the external medium induces a large entry of Ca^{2+} which is inhibited by Gd^{3+} (data not shown). But do the channels studied here function as store-operated channels *in vivo*? The following experiments were designed to examine the level of store-depletion necessary to activate voltage-independent Ca^{2+} channels. Ca^{2+} measurements and patch-clamp studies were performed in parallel on cells maintained in Krebs medium that were first transferred to potassium aspartate medium. Depolarization in this solution induced a $[Ca^{2+}]_i$ transient that was taken as an index of the amount of releasable Ca^{2+} (Fig. 5A, procedure no.1). Fibres were then kept for 5 min in this solution in the absence or in the presence of $1 \mu M$ thapsigargin (Fig. 5A and C, procedures no.2 and no. 4); alternatively, fibres were repolarized rapidly after the first peak of Ca^{2+} and TG was applied for 5 min in a Krebs solution (Fig. 5B, procedure no.3). Whatever the treatment, the fibres were then rinsed in Krebs medium (to prepare for the next depolarization) containing $50 \mu M$ EGTA to avoid any refilling of the stores during this period of time. The return to potassium aspartate solution produced a second peak of $[Ca^{2+}]_i$ the amplitude of which could be compared with the initial transient of $[Ca^{2+}]_i$ in order to estimate the decrease of releasable Ca^{2+} staying in the stores. Compared with the control situation, these three different procedures significantly reduced the content of releasable Ca^{2+} to 69, 34 and 18% of their initial content (Fig. 5D). The level of activity of Ca^{2+} channels was studied under similar conditions and a relationship between the content of releasable Ca^{2+} versus the P_o of store-operated channels is presented in Fig. 5D. It turns out that a threshold of 30% of depletion seems sufficient to activate store-operated channels.

Functional role of SOCs/SACS in muscle fatigue

Muscles stimulated maximally and repeatedly present a progressive decrease in tension production, in shortening velocity and in relaxation speed. These observations, commonly grouped under the concept of muscle fatigue, were classically attributed to the accumulation of intracellular lactic acid (Hill & Kupalov, 1929) resulting in acidosis, but it is now clear that this effect contributes little to muscle fatigue (Allen, 2004) and that intracellular ionic changes also play major roles in this process (see Discussion; Stephenson *et al.* 1998). The following experiments were designed to

paired *t* test (for comparisons of open probabilities and quantities of charge). *D*, dose-response relationship of the inhibition of TG-induced currents (the number of patches under each condition is indicated; s.e.m. is smaller than symbols). *E*, response (in percentage) to $1 \mu M$ TG in the absence or in the presence of $50 \mu M$ Gd^{3+} , $50 \mu M$ La^{3+} , $100 \mu M$ 2-APB and $30 \mu M$ SKF-96365. *Significantly different ($P < 0.05$) from control conditions (TG without inhibitors; one-way ANOVA followed by a Tukey test).

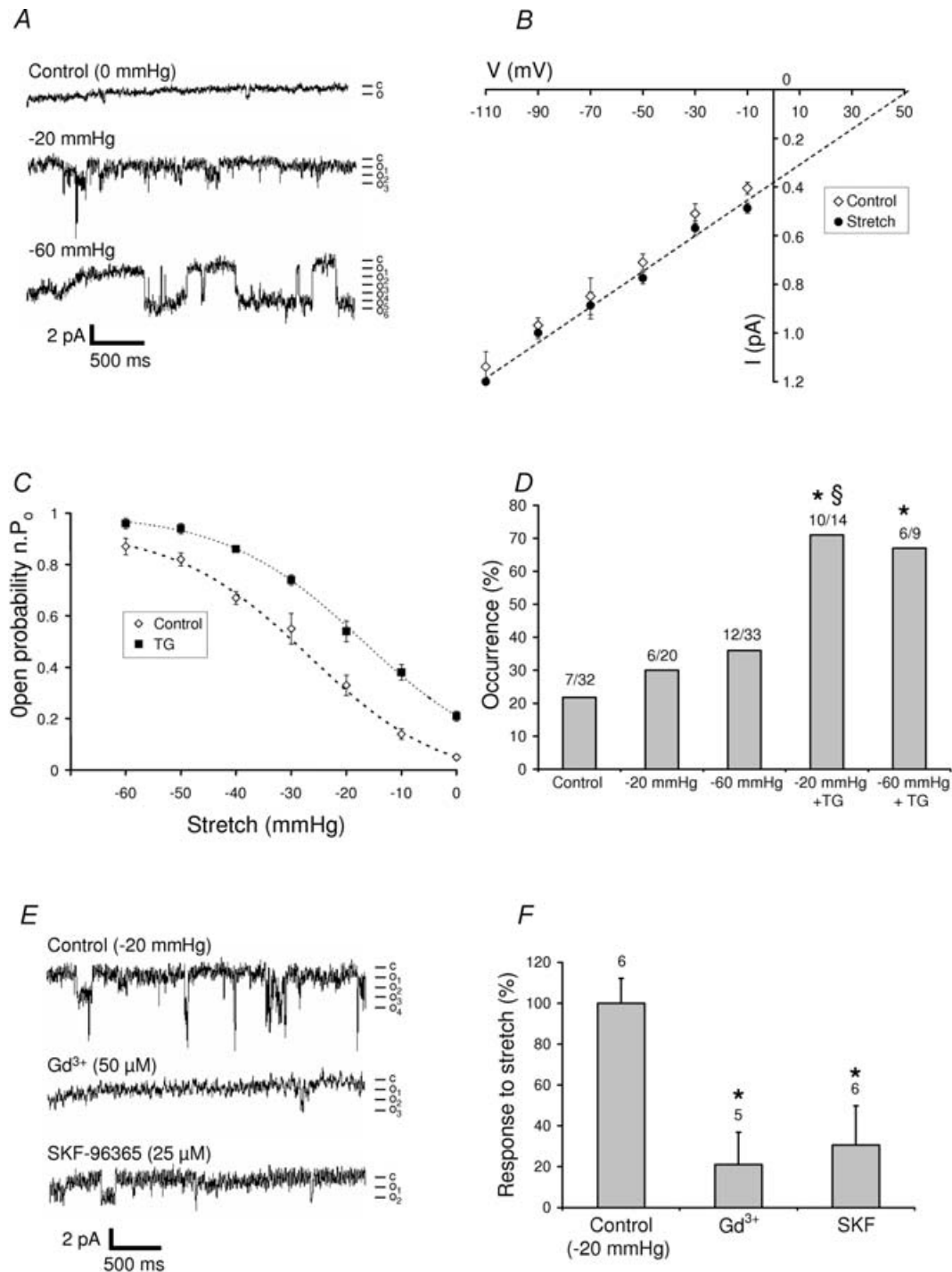


Figure 2. Characterization of stretch-induced Ca^{2+} currents in skeletal muscle fibres

A, examples of current traces (patch clamp; cell-attached configuration at -60 mV holding potential) recorded before (control) and after the indicated pressures applied to the patch electrode (same patch recorded). Mean unitary currents of 0.84 pA. c, closed state; o, open states. *B*, current–voltage relationships in control (\diamond) and stretch-activated (\blacksquare) cells. Voltages indicated were determined from the applied holding potential and the estimated resting potential (-50 mV). *C*, effects of the pressure level of suction applied to the patch electrode on $n.P_o$ in the absence (\diamond , $n = 3$) or in the presence (\blacksquare , $n = 5$) of $1 \mu\text{M}$ TG. Boltzmann fit (dashed line). *D*, occurrence of channel activity recorded under control conditions, during stretches (-20 and -60 mmHg suction), and during the same stretches but after 5 min application of $1 \mu\text{M}$ TG. *Significantly different ($P < 0.05$) from control (χ^2 test, 4 degrees of freedom; results then compared two-by-two, χ^2 test or Fisher exact tests). Significantly different ($P < 0.05$) from -20 mmHg pressure (Fisher exact test). The proportion of patches in which an activity is

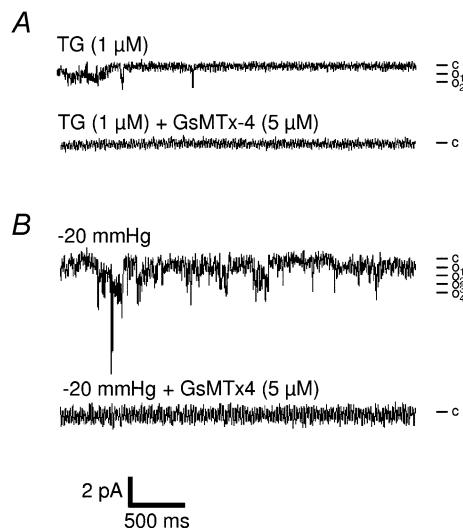


Figure 3. Effects of GsMTx4 on TG- and stretch-induced Ca^{2+} currents in skeletal muscle fibres

Representative records of current traces (patch clamp; cell-attached configuration at -60 mV holding potential) recorded in the absence and in the presence of $5 \mu\text{M}$ GsMTx4, after stimulation by $1 \mu\text{M}$ TG (A) and during a -20 mmHg suction (B). Mean unitary current of 0.61 pA. c, closed state; o, open states.

assess the role of the influx of Ca^{2+} in muscle fatigue. Soleus muscles were chosen for their dependence on oxidative metabolism, thus limiting the role of anaerobic processes in ionic changes and their contribution to muscle fatigue. These muscles were subjected to 50 Hz stimulation trains of 500 ms duration at 1 s intervals (50% duty cycle). After 2 min, the muscles were allowed to recover and were stimulated at 10 s intervals for 10 min. Under control conditions, maximal force was maintained during the first 30 s and then progressively declined. At the same time, relaxation became incomplete during the 0.5 s separating two successive stimulation periods (Fig. 6A). Recovery occurred during the first 5 min after the protocol of fatigue. This recovery was complete and a second fatigue protocol could be performed without any modification in comparison to the first protocol. In the absence of extracellular Ca^{2+} , the maximal force was not maintained, declined significantly faster and the relaxation stayed complete during the whole protocol (Fig. 6A). Recovery was significantly slower and less complete (data not shown). In order to investigate the possible involvement of the store-dependent and mechanosensitive channels, the same protocol was followed in the presence of extracellular

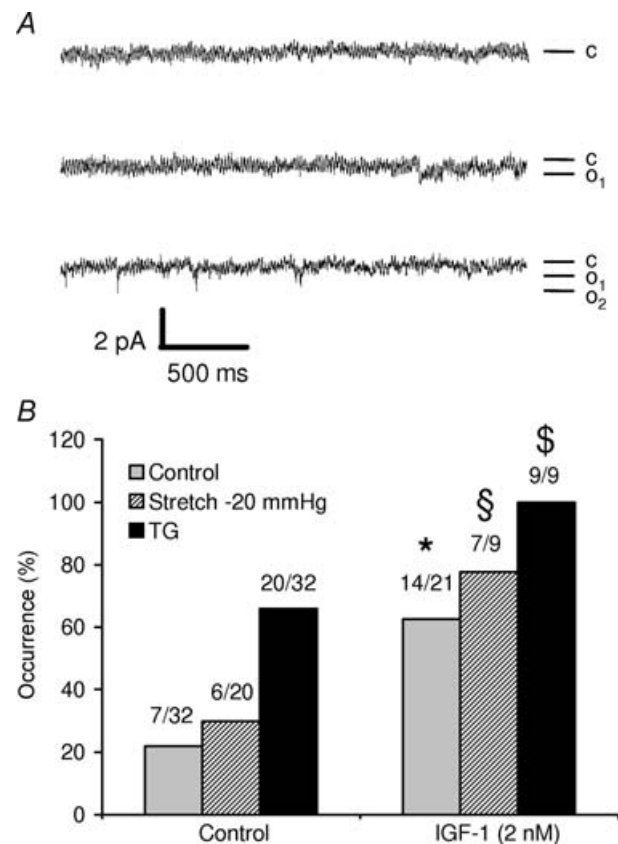


Figure 4. Effects of IGF-1 on TG- and stretch-induced Ca^{2+} currents in skeletal muscle fibres

A, representative records of current traces (patch clamp; cell-attached configuration at -60 mV holding potential) recorded 2 min (upper trace), 2.5 min (middle trace) and 3 min (lower trace) after stimulation with 2 nM IGF-1. Mean unitary currents of 0.6 pA. c, closed state; o, open states. B, effects of 5 min stimulation with 2 nM IGF-1 on the channel activity measured at rest, after a stretch of -20 mmHg or after stimulation with $1 \mu\text{M}$ TG. *Significantly different from control ($P < 0.05$, χ^2 test); §significantly different from the -20 mmHg stretch in the absence of IGF-1 ($P < 0.05$, χ^2 test); \$significantly different from the stimulation with TG in the absence of IGF-1 ($P < 0.05$, Fisher exact test). The proportion of patches in which an activity is detected is indicated above each bar.

Ca^{2+} but in the absence and then in the presence of SKF-96365, Gd^{3+} or GsMTx4 toxin (paired experiments). The presence of Gd^{3+} ($50 \mu\text{M}$) significantly accentuated the decrease of force during the fatigue protocol (Fig. 6B; $P < 0.05$, t test on paired experiments). The presence of SKF-96365 also accentuated the decrease of force albeit a little later (effect significant after 30 s). Finally, the GsMTx4 toxin ($5 \mu\text{M}$; tested only two times because of its cost)

detected is indicated above each column of the histogram. E, pharmacological characterization of stretch-activated single-channel currents. Examples of current traces (patch-clamp; cell-attached configuration at -60 mV holding potential) recorded under control condition (during a -20 mmHg suction) or in the presence of Gd^{3+} ($50 \mu\text{M}$) and SKF-96365 ($25 \mu\text{M}$). Mean unitary currents of 0.87 pA. F, response (in percentage) to -20 mmHg stretch in the absence and presence of $50 \mu\text{M}$ Gd^{3+} or $30 \mu\text{M}$ SKF-96365. *Significantly different ($P < 0.05$) from control conditions (stretch without inhibitors; one-way ANOVA followed by a Tukey test).

had a similar effect and partially mimicked the absence of extracellular Ca^{2+} (Fig. 6B).

Discussion

In the present paper, we show that adult muscle fibres have SACs and SOCs that share several biophysical and pharmacological properties. Indeed, they present the same unitary conductance, support currents having the same reversal potential and have a similar sensitivity to Gd^{3+} , SKF-96365, 2-APB and GsMTx4 toxin. Besides, the occurrence of both channels is increased after IGF-1 stimulation. Together, these observations might suggest that they belong to the same population or share common constituents.

Possible molecular identity of SOCs and SACs

SACs in muscle fibres might be constituted of the TRPC1 isoform, which has been shown to form such stretch-activated cation channels in vertebrate cells (Maroto *et al.* 2005). Accordingly, this channel is

completely inhibited by the spider venom toxin GsMTx4, which, to date, has only been shown to block SACs encoded by TRPC1 (Suchyna *et al.* 2000; Gottlieb *et al.* 2006). We have previously suggested the involvement of this protein as a constituent of SOCs in normal and dystrophic skeletal muscle fibres (Vandebrouck *et al.* 2002). The similarity of the properties of SACs and SOCs, in particular their similar sensitivity to GsMTx4 toxin, suggests that both channels might be constituted, at least partially, of TRPC1 protein. Similarly, SACs and SOCs might also involve TRPV2 protein (previously named GRC or VRL1), a channel which translocates to the membrane upon IGF-1 stimulation and which is elevated in the membrane of dystrophic patients (Kanzaki *et al.* 1999; Iwata *et al.* 2003; Muraki *et al.* 2003; Yeung *et al.* 2005). Finally, they might implicate the TRPV4 isoform which is activated by cell swelling through a phospholipase A_2 (PLA_2)-dependent mechanism (Vriens *et al.* 2004). Interestingly, we have previously shown that the channels described here have an abnormally high open probability in dystrophic muscle fibres (Gailly, 2002; Vandebrouck *et al.* 2002), a situation in which the activity of PLA_2 has been reported to be increased by a factor of 40 (Lindahl *et al.* 1995).

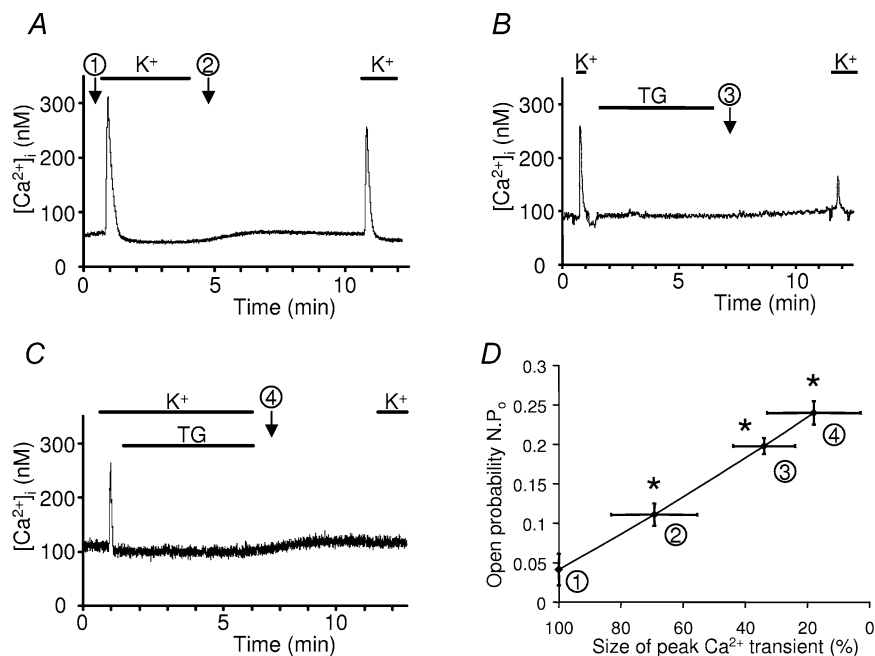


Figure 5. Level of store depletion and activation of voltage-independent Ca^{2+} channels

A, B and C, depolarization in potassium aspartate solution induced a $[\text{Ca}^{2+}]_i$ transient that was taken as an index of the amount of releasable Ca^{2+} (procedure no. 1). Fibres were then kept for 5 min in potassium aspartate solution in the absence or in the presence of $1 \mu\text{M}$ TG (A and C, procedures no. 2 and no. 4); alternatively, fibres were repolarized rapidly after the first peak of Ca^{2+} , and TG was applied for 5 min in a Krebs solution (B, procedure no. 3). After repolarization in Krebs medium (5 min), the amount of releasable Ca^{2+} was re-estimated (see details in the text). D, relationship between the P_o measured by patch-clamp (at the time and under the conditions indicated by the encircled numbers) and the amount of releasable Ca^{2+} from the stores in C57 mice muscle fibres. *Significantly different from control ($P < 0.05$): sizes of peak Ca^{2+} transients compared by a *t* test (paired experiments); P_o values were compared by a one-way ANOVA followed by a Tukey test. Procedures are indicated by encircled numbers. K^+ , potassium aspartate solution.

Precise identification of the isoform(s) involved is under study.

Functional role of SACs and SOCS in skeletal muscle fibres

It is not obvious that store depletion occurs *in vivo* because adult muscle fibres do not exchange much Ca^{2+} with the extracellular medium. Indeed, skeletal muscle fibres have huge amounts of sarcoplasmic reticulum which is extremely rich in Ca^{2+} -pumps (SERCA) and which contains a high buffering capacity (calsequestrin). Ca^{2+} extrusion through the plasma membrane seems also very slow (reviewed in Martonosi & Pikula, 2003). So almost all of the Ca^{2+} released from the sarcoplasmic reticulum

is rapidly restored to the sarcoplasmic reticulum after stimulation; accordingly, twitch contractions can thus be produced repeatedly in the absence of extracellular Ca^{2+} (Armstrong *et al.* 1972). However, evaluations of Ca^{2+} influx using the Ca^{45} uptake technique indicate that each twitch contraction induces a small increase of Ca^{2+} entry (Bianchi & Shanes, 1959), the mechanism of which is unknown. Here, we show that a decrease of the order of 30% of the Ca^{2+} stores is sufficient to induce an entry of Ca^{2+} . Interestingly, it has been evaluated that a single action potential triggers the release of 0.2–0.3 mM from the sarcoplasmic reticulum to the cytoplasm (Baylor *et al.* 1983), which corresponds to more than a quarter of the Ca^{2+} contents present in the sarcoplasmic reticulum (Endo, 1977). Thus it seems reasonable to

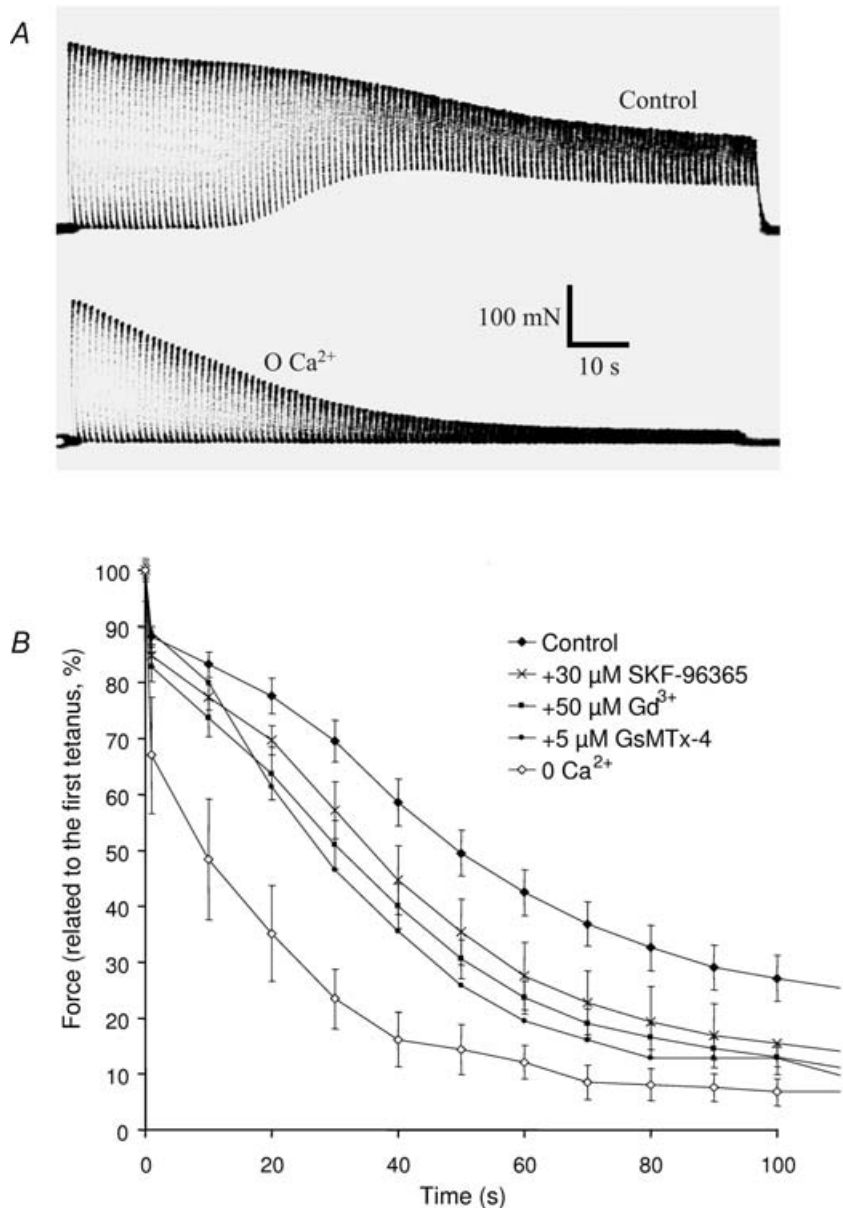


Figure 6. Involvement of Ca^{2+} entry in muscle fatigue

A, representative examples of force records. Muscles stimulated in the presence (upper panel) or in the absence (lower panel) of Ca^{2+} . Tetani of 500 ms every second for 2 min (50 Hz stimulation frequency). B, quantification of the loss of force during the protocol of fatigue (force measured every 10th tetanus). Results expressed relative to the maximal force produced during the first tetanus. Five paired experiments performed for each condition, except for GsMTx4 toxin ($n = 2$). Statistical significance indicated in the text.

think that store-operated channels are indeed activated during *in vivo* contraction. This is corroborated by the fatigue experiments. In the absence of external Ca^{2+} or when SOCs are inhibited, a faster decline of force is observed, suggesting that a sustained activity, such as the one observed in tonic muscles as soleus, requires a constant repletion of the stores by a subsequent entry of Ca^{2+} . A partial failure of the sarcoplasmic reticulum to release Ca^{2+} during tetanus has been proposed as a possible cause of fatigue (Allen & Westerblad, 2001; Allen, 2004). This seems to be due to the accumulation of Ca^{2+} phosphate in the sarcoplasmic reticulum (Kabbara & Allen, 1999; Dutka *et al.* 2005). It is interesting to note that, at rest, the sarcoplasmic reticulum of slow twitch fibres (as in soleus muscle) is saturated with Ca^{2+} while the sarcoplasmic reticulum of fast twitch muscle fibres is only about one-third full (Fryer & Stephenson, 1996), suggesting that the amount of Ca^{2+} in the stores might be more critical for the physiological function of slow twitch fibres. Our results emphasize the importance of the entry of Ca^{2+} during sustained trains of contractions of slow twitch muscle. Such importance of Ca^{2+} handling in muscle fatigue has also been suggested in fast and slow muscles deficient in mitsugumin, a protein expressed at the triad junction, the lack of which leads to a disorganization of the T-tubules and sarcoplasmic relationship and to a susceptibility to fatigue and to a dysfunction of store-operated entry of Ca^{2+} (observed in embryonic and neonatal muscles but not in adult fast fibres) (Nishi *et al.* 1999; Nagaraj *et al.* 2000; Kurebayashi & Ogawa, 2001; Kurebayashi *et al.* 2003; Ma & Pan, 2003).

Whether the channels studied here are also activated by stretch during contraction is difficult to evaluate. Indeed, the force applied to membrane patches in the experiments presented here represents only about 5% of the force developed by muscle during contraction. However, there are no data on the possible transmission of force (produced during contraction) to the cell membrane.

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