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ORIGINAL PAPER

Extracellular stimulation with human "noisy" electromyographic patterns facilitates myotube activity

M. Sciancalepore[1] · T. Coslovich[2] · P. Lorenzon[1] · G. Ziraldo[1] · G. Taccola[2]

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Abstract Electrical stimulation (ES) of skeletal muscle partially mimics the benefits of physical activity. However, the stimulation protocols applied clinically to date, often cause unpleasant symptoms and muscle fatigue. Here, we compared the efficiency of a "noisy" stimulus waveform derived from human electromyographic (EMG) muscle patterns, with stereotyped 45 and 1 Hz electrical stimulations applied to mouse myotubes in vitro. Human gastrocnemius medialis electromyograms recorded from volunteers during real locomotor activity were used as a template for a noisy stimulation, called EMG*stim*. The stimulus-induced electrical activity, intracellular Ca^{2+} dynamics and mechanical twitches in the myotubes were assessed using whole-cell perforated patch-clamp, Ca^{2+} imaging and optical visualization techniques. EMG*stim* was more efficient in inducing myotube cell firing, $[Ca^{2+}]_i$ changes and contractions compared with more conventional electrical stimulation. Its stimulation strength was also much lower than the minimum required to induce contractions via stereotyped stimulation protocols. We conclude that muscle cells in vitro can be more efficiently depolarized using the "noisy" stochastic stimulation pattern, EMG*stim*, a finding that suggests a way to favor a higher level of electrical activity in a larger number of cells.

Keywords Human electromyogram · Myotube contractions · Perforated patch-clamp recording · Intracellular calcium · Electrical stimulation

Introduction

Physical activity is a benefit, which improves the quality of life not only in young and healthy people, but also in subjects affected by muscle weakness due to ageing, or patients suffering from prolonged debilitating illness or traumatic injury of the central and peripheral nervous system. However, quite often, the elderly and other patients cannot exercise because of physical and/or psychic constraints. Electrical stimulation (ES) of skeletal muscle has been proposed to mimic the beneficial effects of physical training and to counteract the muscle atrophy associated with reduced motor activity. Studies conducted in vitro have shown that ES induces adaptive responses and functional metabolic changes (Nikolić et al. 2012), fiber-type switches (Wehrle et al. 1994; Burch et al. 2010), and satellite cell recruitment/differentiation (Pedrotty et al. 2005; Langelaan et al. 2011), all aspects related to maintenance of muscle trophism.

To date, both in clinics and in laboratory research, the most efficient contraction of myofibers can be evoked by protocols of ES consisting of the delivery of biphasic trains of rectangular impulses, at a frequency of 40–45 Hz (Thrasher et al. 2005; Gomis et al. 2009). However, electrically-evoked contractions inevitably decay, despite the persistence of stimulation (Bickel et al. 2003; Gregory et al. 2005). Therefore, the identification of new and more efficient stimulation protocols is one of the main goals for improving the beneficial effects of the ES.

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✉ M. Sciancalepore
msciancalepore@units.it

[1] Department of Life Sciences & BRAIN Center, University of Trieste, Via Fleming 22, 34127 Trieste, Italy

[2] Department of Neuroscience, SISSA, Trieste, Italy

In general, ES with asynchronous stimuli, better recalls the intrinsic variability of the activity profile of a cellular population (Martínez et al. 2007; McDonnell and Abbott 2009; Ziegler et al. 2010) and may better improve the recruitment of excitable cells than the delivery of stereotyped stimuli (Taccola 2011; Dose and Taccola 2012). Electromyogram (EMG) recordings from human muscles represent a practical and readily available source of realistic variable impulses, that can be used as a template to evoke the activation of excitable cells (Dose et al. 2013). In more detail, a segment of EMG recording sampled from a limb muscle of an adult volunteer during the execution of a rhythmic motor activity, could potentially represent one of the more physiological electrical patterns for efficient muscle stimulation.

The aim of the present study was therefore to assess whether "noisy" ES protocols, obtained by sampling human EMG recordings and delivered to cultured muscle cells, might be more convenient and efficient for inducing contractile activity, compared to the canonical protocols of stereotyped stimulations. To this purpose, clinical human EMG recordings were combined with perforated whole cell patch-clamp recordings and videoimaging of murine myotubes in culture in order to compare the effects of "noisy" with that of more common tetanic and 1 Hz, low-frequency stimulation protocols.

Materials and methods

Primary muscle cell cultures

Primary myoblast cultures of i28 skeletal muscle cells were established from mouse satellite cells, kindly provided by Prof. Wernig, University of Bonn, Germany (Irintchev et al. 1997). Cells were derived from the hind-leg muscles of a 7-day-old male Balb/c mouse killed by cervical dislocation as approved by Local Animal Care Committee and in agreement with the European legislation. Undifferentiated myoblasts were maintained as exponentially growing myoblasts in Growth Medium consisting of Ham's F10 supplemented with 20 % fetal calf serum, 4 mM L -glutamine, 100 U/ml penicillin, and (100 µg/ml streptomycin) and sub-cultured by standard trypsinization every 3 days. To induce differentiation, 24 h after plating, the growth medium was replaced with a differentiation medium with a serum content decreased to 2 %. For all studies, muscle cells were seeded at 70,000 cells/35 mm on matrigel-coated Petri dishes (BD Biosciences, San Jose, CA, USA). Cultures were maintained at 37 °C and 5 % CO_2. The medium was renewed every 3 days to avoid loss of nutrients and growth factors. All the experiments were performed in 6-8 day old myotubes.

EMG recordings during locomotion

EMG Ag/AgCl surface electrodes (10 mm diameter, 21 mm inter-electrode distance) were positioned on the right gastrocnemius medialis muscles of three different adult volunteers during overground locomotion at a freely selected speed ($\sim$1 m/s) and connected to the TELEMG® system (BTS, Milano, Italia) for recording muscle electric activity.

Patterns of electrical stimulations on cultured muscle cells

Electrical field stimulation of skeletal myotubes in culture was performed giving bipolar pulses with platinum-iridium electrodes (0.2 mm diameter) placed within 3 mm of each other and positioned 1–2 mm over the cells. They were programmed by computer-based control software and delivered by the stimulator (STG 4002®; Multi Channel Systems, Reutlingen, Germany). Concentric bipolar electrodes had the inner pole inserted in a glass pipette filled with physiological extracellular solution surrounded by the outer pole arranged as a wire coil hanging in the bath solution.

A new protocol, named EMG *stimulation* (EMG*stim*), was obtained by elaborating EMG signals. They were notched at 50 Hz, band-pass filtered with cut-off frequencies from 5 to 200 Hz and amplified × 1000. Epochs (60 s) of such EMG signals were sampled at 500 Hz with Clampfit® 10.3 software (Molecular Devices, USA) and exported as ASCII text files to a multichannel programmable stimulation device (STG 4002®), working with an output current resolution of 2 µA, a time resolution of 20 µs and an output current slope of 600 µA/µs. With the same stimulator, stereotyped trains of pulses at 45 or 1 Hz were performed using various intensities (1–6 V peak to peak). Single pulses (width = 1 ms) were delivered as cathodic-first charge-balanced biphasic rectangular current injection without a delay between the cathodal and anodal phases. A 60 s segment of Gaussian noise was also artificially created by Clampfit 10.3 software for use in comparison with EMG*stim* or stereotyped pulses.

Electrophysiological recordings

Characterization of the electrical membrane properties of the myotubes was performed with whole cell patch-clamp techniques, by recording the changes in membrane potential under current-clamp conditions using an Axopatch 200B® amplifier (Axon Instrument, Foster City, CA, USA). Voltage recordings were sampled at 100 kHz and low-pass filtered at 2 kHz. Later, in order to maintain the same

sampling frequency of the stimulating protocol EMG*stim*, they were sampled at 500 Hz and filtered according to the experimental requirements. The perforated-patch clamp method was preferred to conventional whole-cell recording in order to allow exchange of small ions only across the muscle cell membrane and to avoid washout of important intracellular messengers. Extracellular recording solution (NES) contained (in mM): 100 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH = 7.3 at room temperature (22–24 °C). Pipette solution contained (in mM): 140 K-aspartate, 10 NaCl, 2 $MgCl_2$, 10 HEPES, pH 7.3 and amphotericin B (150 μg ml^{-1}). An agar salt bridge was used to minimize the junction potential between the Ag/AgCl electrode and the pipette solution. Mouse myotubes that occasionally exhibited spontaneous contractions, were discharged.

Assessment of cell contractions

To detect myotube twitching, cell images were collected by a CCD camera (SensiCam®; PCO Computer Optics, Kelheim, Germany) mounted on an inverted phase-contrast microscope (Axiovert S100, Carl Zeiss, Jena, Germany) with a 40 × objective. Sampling was 17 images/s.

Ca^{2+} imaging

Intracellular calcium $[Ca^{2+}]_i$ changes were monitored in muscle cells plated on glass coverslips coated with matrigel, loaded with the fluorescent Ca^{2+}-sensitive dye fura -2. Cell loading consisted of 30 min cell incubation, in the dark, with 5 μM fura 2-pentaacetoxymethyl ester in NES supplemented with 1 % Bovine Serum Albumin. After loading, Ca^{2+} transients were monitored in cells maintained in NES at 37° C and excited alternately at 340 and 380 nm. Fluorescence images were acquired by a CCD camera (IMAGO CCD camera, Till Photonics, Gräfelfing, Germany) at a rate of 12 images/s, using an oil immersion 40x objective. Image acquisition and off-line processing were done by an integrating imaging software package (TILLvisION, Till Photonics). $[Ca^{2+}]_i$ transients were measured as variations in the mean value of the fluorescence intensity in ratio images (340/380). The fluorescence ratio at rest was assumed to be 1. In the temporal plots (fluorescence ratio in areas of interest *vs* time), the $[Ca^{2+}]_i$ increase was expressed as an increase in fluorescent signal relative to the fluorescence at rest. The percentage of responsive myotubes was calculated in each optical field as the number of myotubes exhibiting $[Ca^{2+}]_i$ transients out of the total number observed. All the experiments were performed on 7 independent cell culture preparations observing at least 30 different optical fields.

Chemicals

If not otherwise specified, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Statistical analysis

All data are expressed as mean ± S.D., unless otherwise specified; "n" indicates the number of experiments. Before assessing statistical differences among groups, a normality test was performed to select the use of either parametric or non-parametric tests. To compare more than two sets of non-parametric values, Kruskal–Wallis ANOVA on ranks, followed by post hoc analysis with Tukey's method was applied. Collected results were considered significant when $P < 0.05$.

Results

Delivery of "noisy" waveforms elicits repetitive muscle cell firing

The stimulating protocols EMG*stim* were composed of noisy waveforms (mean period = 1.11 ± 0.13 s; period CV = 0.08 ± 0.01) and were designed off-line from segments (60 s) of right gastrocnemius medialis electromyograms (EMG) recorded from three adult human volunteers during sessions of real locomotion (Fig. 1a). Figure 1 shows how a representative episode of the EMG recorded from gastrocnemius medialis (grey box in Fig. 1a), when extracellularly delivered to the cultured myotubes at the site indicated by the stimulating pipette in Fig. 1b, elicited myotube excitation. The inset in Fig. 1a shows an expanded part of EMG*stim* to show more clearly the exact nature of the EMGstim pattern that we used. It may be seen that the pattern derived from the human muscle during locomotion in fact consisted of relatively regular bursts of activity interspersed with periods of non-activity. Within each burst, there was a stochastic pattern of varying amplitude and frequency. The transmembrane voltage response to EMG*stim*, recorded in perforated patch configuration from a single myotube at −65 mV membrane potential, is shown in Fig. 1c.

In order to determine the stimulus artifacts potentially contaminating the electrophysiological recordings from myotubes, at the end of each experiment, the same EMG*stim* pattern was repeated with the stimulating electrode raised and positioned above the cells, although remaining inside the recording bath (Supplementary Fig. 1a). In this case, amplitude voltage responses were detected that most likely originated from the propagation of electromagnetic waves arising from the delivery of AC stimulating signals,

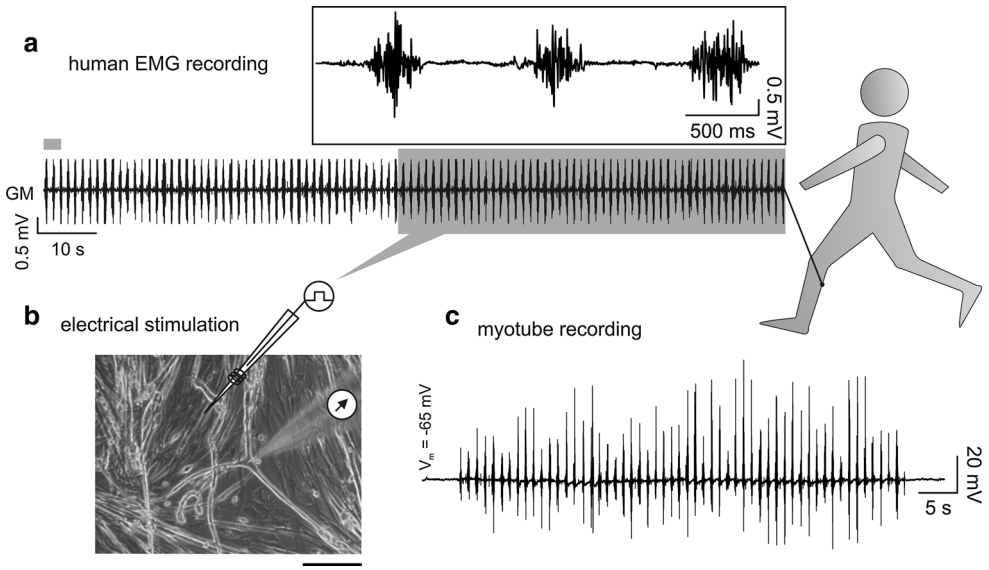

Fig. 1 A 60 s segment trace (*grey box*), sampled from a human EMG recording is taken in order to design off-line, a noisy stimulation pattern, named EMG*stim*. The *inset* shows an expanded part of the EMG*stim* (corresponding to *upper gray rectangle*) to reveal the stochastic nature of the activity bursts. **a** Extracellular delivery of EMG*stim* at a nearby position **b** triggered the intracellular electrophysiological response shown in **c**, recorded from a single myotube under perforated whole-cell patch clamp conditions. *Scale bar* 150 μm. The cell resting potential (V_m) was −65 mV

inducing capacitive and inductive coupling into the recording system (Prior et al. 1993). In Fig. 2, power spectra are shown to detect the main frequencies contained within the stimulation protocol EMG*stim* (a, a_1) itself, as well as the myotube voltage changes in response to EMG*stim* when the stimulating electrode was positioned close to the myotubes (b, b_1) or far from them, in the bath (c, c_1). The power spectrum of the stimulus artifact interference (inset in Fig. 2c_1) showed no frequency components in the 0.1–5 Hz range, whereas the main frequencies recorded when the myotubes were stimulated nearby were around 1–3 Hz (inset in Fig. 2b_1). By filtering the myotube voltage response to EMG*stim* with a band-pass filter within the 0.1–5 Hz range, a series of action potentials was clearly detected. Interestingly, despite some failures, each cycle of EMG*stim* oscillatory activity induced cumulative cell depolarization leading to the generation of action potentials, time-locked with the stimulation waveform, that were inhibited by the specific sodium channel blocker, tetrodotoxin (TTX, 1 μM) (Fig. 2d). By recording the mere interference (Supplementary Fig. 1b) by raising the stimulating electrode far from cells, when the same band-pass filter 0.1–5 Hz was applied, the signal was completely suppressed (Supplementary Fig. 1b_1). Similar results were invariably observed in all the recordings (n = 15) confirming that the filtering band used abolished the interference. In addition, to verify that the filtering settings did not affect the spiking activity, episodes of firing were recorded from spontaneously active myotubes in culture (Supplementary Fig. 1c). Although a reduction in amplitude of action potential peaks was observed (Supplementary Fig. 1c_1), the possibility to still detect the original frequency of action potentials provided us with the rationale to process all the electrophysiological responses to the operative 0.1–5 Hz band-pass filtering.

A random Gaussian noise stimulation pattern was also tested in the same myotube and at the same intensity of EMG*stim*, to see whether it would induce any activation of muscle cell electrical activity (n = 8 cells); however, such a random stimulation was not sufficient to produce cell firing (Fig. 3).

EMG*stim* has a high efficiency in inducing firing

Repeated tetanic stimulations with high-frequency electrical 1 ms pulses at 45 Hz induced in myotubes an electrical activity invariably characterized by failures, detected as discontinuous firing preceding the complete disappearance of the electrical activity (Fig. 4, n = 6). The main frequencies recorded when the myotubes were electrically stimulated by EMG*stim* were in the range of 1–3 Hz (inset in Fig. 2b_1) and occasionally present spontaneous activity of myotubes was around 1 Hz (Sciancalepore et al. 2005), therefore, EMG*stim* versus Low frequency stimulation at 1 Hz was tested, assuming that the low frequency represented a more physiological stimulation pattern. In more detail, the voltage changes induced by stimulation at 1 Hz and EMG*stim* were compared in the same skeletal myotubes. The application sequence of the two stimulation protocols were counter-balanced to eliminate any

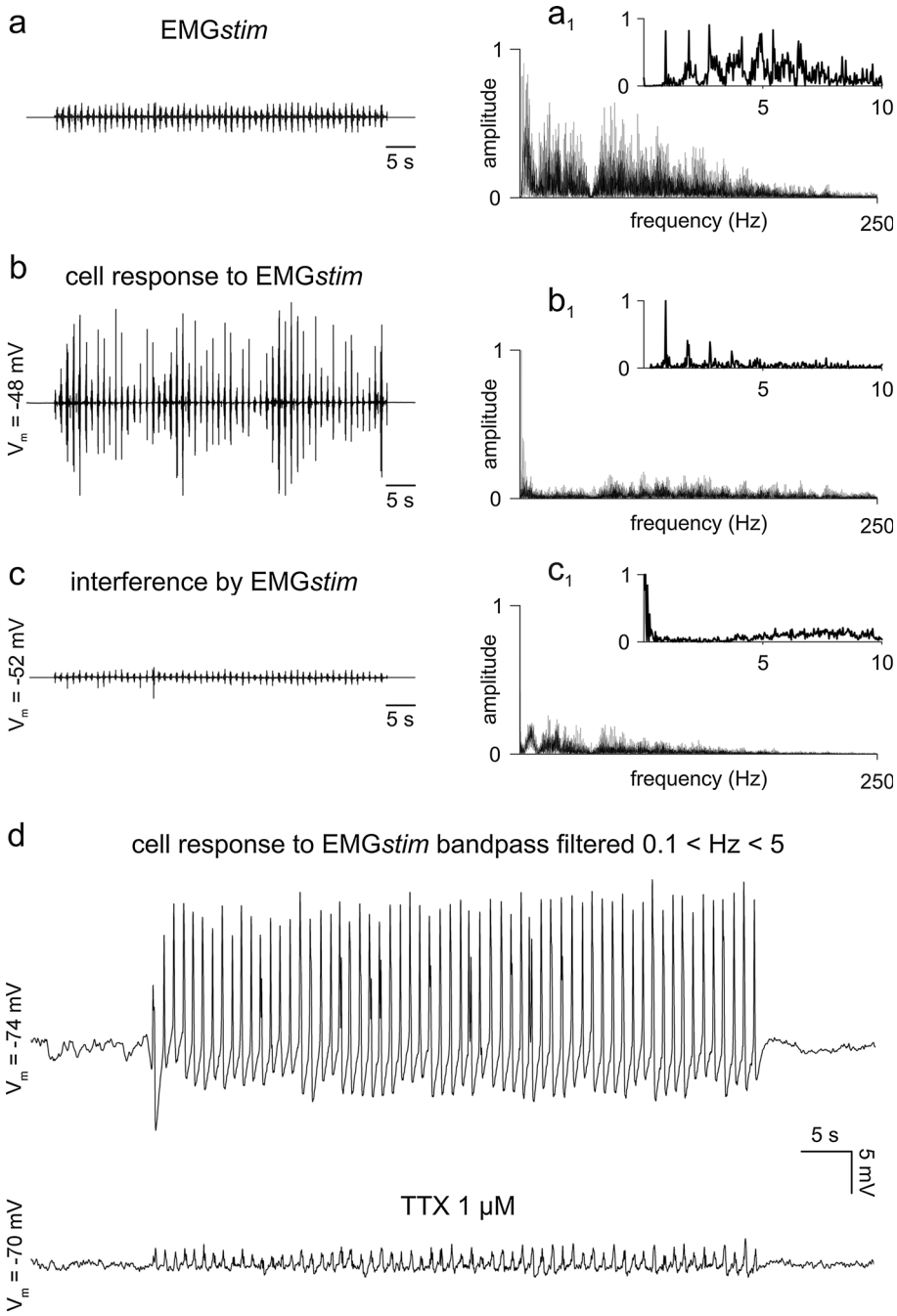

Fig. 2 Power Spectral analysis of voltage frequency components of the EMG*stim* stimulus pattern alone (**a**, **a$_1$**), the intracellular voltage response to EMG*stim* recorded in a single myotube (**b**, **b$_1$**) and the stimulus artifact interference induced in the cell by EMG*stim* applied at a distant location (**c**, **c$_1$**). Normalized Fast Fourier Transform amplitude is shown at the ordinates. The insets show the low frequency distributions in detail. No components in the 0.1–5 Hz range are present in the interference (**c$_1$**) whereas, 1–3 Hz frequencies predominate in the voltage response to EMG*stim* (**b$_1$**). When it was band-pass filtered at 0.1–5 Hz, although failures occur, TTX-sensitive action potentials appeared to be time-locked with the EMG*stim* pattern (**d**)

systematic effect due to the order of testing. Low frequency 1 ms pulse electrical stimulations at 1 Hz induced individual action potentials time-locked with the electric pulses (Fig. 5a) and mechanical contractions. In these electrophysiological experiments, the lowest intensity of 1 Hz stimulation able to elicit a detectable myotube twitching was defined as "twitching stimulation", Tw*stim*. The mean Tw*stim* value, detected in 22 optical fields in 7 different cell cultures, was 4.4 ± 0.2 V (voltage range 2.2–6 V). A representative trace of rhythmic action potentials elicited at 6 V for 60 s is shown in Fig. 5a, whereas no action potentials were recorded when such protocol was applied at 3 V (Fig. 5a, lower panel, n = 22). In Fig. 5b EMG*stims* obtained from the electromiography of three different individuals were applied to the same myotube then in a, at 3 V (Tw*stim* ½). The lowest intensity (peak to peak) of EMG*stim* able to elicit a detectable myotube twitching was on average = 2.2 ± 0.1 V (voltage range 0.7–3 V) detected in 29 optical field in 7 different cell cultures. Such a "noisy" stimulation protocol was found to be more

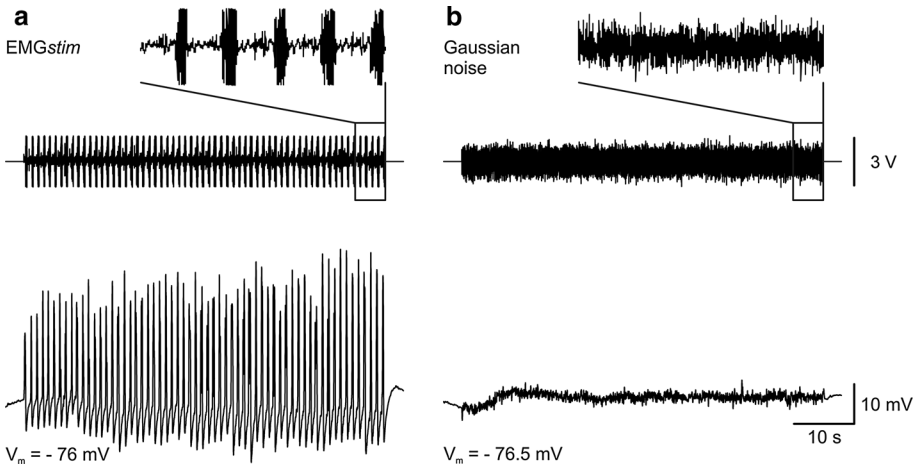

Fig. 3 Representative voltage responses elicited in a myotube by EMG*stim* (**a**) or a random Gaussian noise stimulation (**b**) delivered at 3 V in the same cell. The traces are band-pass filtered at 0.1–5 Hz. The insets show the stimulation patterns in detail. Note that EMG*stim* was more efficient in inducing muscle action potential firing than the continuous random Gaussian noise pattern

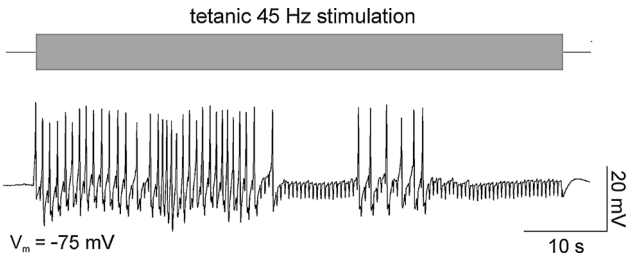

Fig. 4 Representative firing elicited by a biphasic tetanic stimulus at 45 Hz delivered at 6 V. The trace is band-pass filtered at 0.1–5 Hz

efficient in inducing action potential firing and contractions at half of the intensity required for 1 Hz stimulation.

EMG*stim* facilitates the activation of a larger number of muscle cells

Firing activity of myotubes was always associated with contractions, as revealed by observing twitching myotubes in representative optical fields during ES. As in muscle fibres, myotube contraction is mediated by $[Ca^{2+}]_i$ increase, flowing into the cytoplasm mainly from the sarcoplasmic reticulum. To quantify the percentage of myotubes able to twitch following specific ES, Ca^{2+} transients elicited by the electrical stimulation were monitored by Ca^{2+} imaging. In Fig. 6, representative Ca^{2+} transients are shown relative to three representative myotubes in the same optical field, stimulated with different protocols. In this subset of experiments, the maximum peak-to-peak stimulation amplitude was adjusted to pre-selected values of 6 and 3 V using the EMG*stim* or 1 Hz protocol. 195 cells were tested in at least 30 optical fields for each stimulating pattern. Interestingly, low-intensity stimuli (3 V) induced Ca^{2+} transients as well as twitches in a large percentage of myotubes when EMG*stim* was applied (mean = 44.8 ± 7.3 % myotubes, n = 42 optical fields), whereas stimulation at 1 Hz was effective only in a few

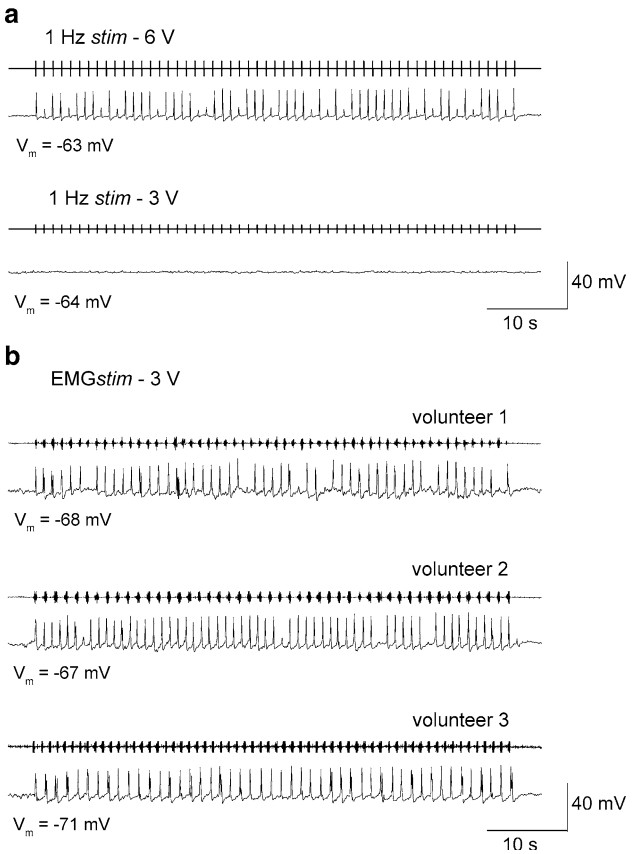

Fig. 5 Biphasic 1 Hz −1 ms stimulations for 60 s induced in a representative myotube, single action potentials at 6 V and no activity at half-stimulation (3 V). However, EMG*stim*s obtained from the electromyogram of three different individuals, given to the same myotube at 3 V, always elicited a constant firing activity

cells (mean = 8.10 ± 3.9 % myotubes, n = 41 optical fields). A number of responsive cells similar to the one elicited by EMG*stim* was observed only when 1 Hz stimulation was delivered at the double intensity level of 6 V (mean = 33.2 ± 9.00 % myotubes, n = 30 optical fields;

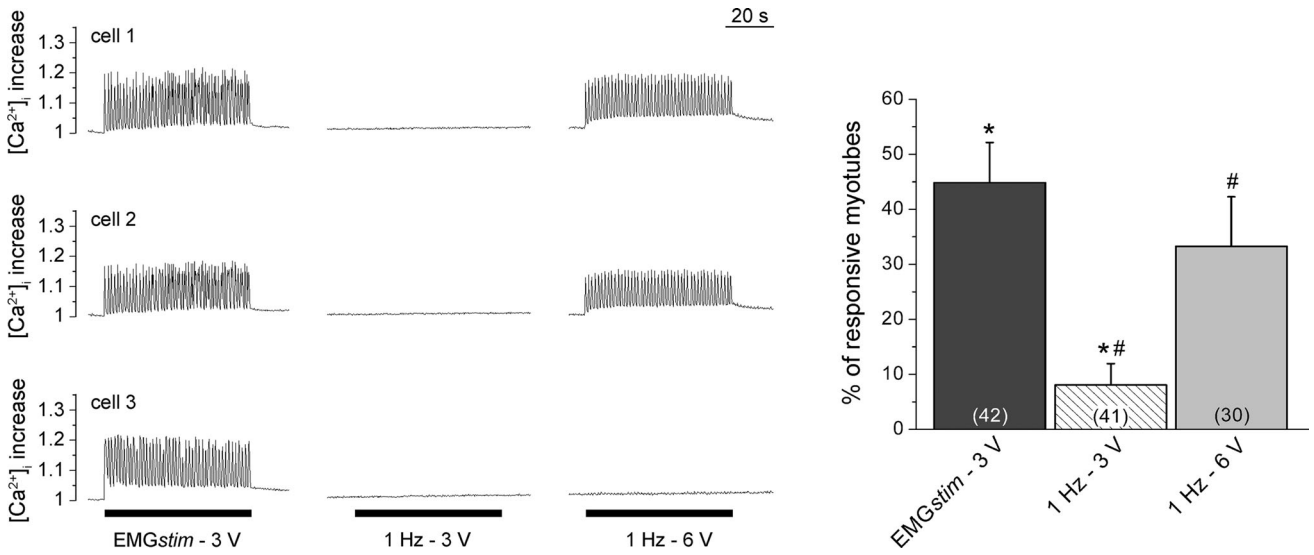

Fig. 6 Representative $[Ca^{2+}]_i$ changes detected in three twitching myotubes during field electrical stimulation protocols (**a**). At 3 V, EMG*stim* elicited $[Ca^{2+}]_i$ transients, whereas 1 Hz stimulation did not, unless the intensity of stimulation was increased to 6 V. The histogram in **b** shows a summary of all the experiments revealing that at 3 V, the percentage of responsive cells to EMG*stim*, was significantly ($p = 0.003$) higher than that observed during the low-frequency stimulation at 1 Hz (* significantly different). At 6 V, 1 Hz stimulation, the number of responsive cells was significantly higher than that observed at 3 V (#) and comparable to that observed with EMG*stim* at 3 V. The number of optical fields used to calculate the mean values is shown in parentheses; the *error bars* indicate SE

Fig. 6b; $P = 0.003$; Kruskal–Wallis One Way ANOVA on Ranks followed by all pairwise multiple comparison with Tukey Test, $n = 7$ cell cultures).

Discussion

Electrical stimulation (ES) has been proposed as a new strategy for physical training as well as to counteract and recover from muscle atrophy. In this work, we demonstrate that using "noisy" patterns of human muscular activity derived from EMG recordings, was more efficient in inducing in vitro muscle cell firing and $[Ca^{2+}]_i$ transients associated with cell contractions compared with stereotyped low-frequency stimulation at 1 Hz and presented minimal failures compared with tetanic 45 Hz stimulations. Our observations therefore make the EMG-like "noisy" ES approach promising, opening a new perspective for the optimization of the stimulation protocols for therapeutic use in patients.

Over the years, ES has been considered a useful technique for partially restoring the activity of paralyzed muscles and for improving circulation in paralyzed limbs after spinal cord injury or in stroke patients (Stein et al. 2002). This was achieved either through surgical implants (Hayashibe et al. 2011) or percutaneous intramuscular electrodes (Agarwal et al. 2003). However, classical ES exhibits several limitations, such as the unpleasant symptoms due to pulse strength (Delitto et al. 1992; Naaman et al. 2000) and the occurrence of muscle fatigue, a sign of neuromuscular failure (Bickel et al. 2003; Gregory and Bickel 2005). Therefore, the most appropriate parameters of stimulation, such as intensity, frequency and pulse duration, are still under debate (Bergquist et al. 2011).

According with the evidence that in neuronal networks, subthreshold noisy stimulation elicited locomotor-like oscillations with high efficiency (Dose and Taccola 2012; Taccola 2011; Dose et al. 2013), we report here that "noisy" EMG*stim* derived from human EMG recordings, was always more efficient than conventional stereotyped protocols and this was not only due to the randomized nature of the stimulation pattern because the Gaussian noise does not induce cell activity. In myotubes in culture, EMG*stim* was able to induce Ca^{2+} transients synchronous to pulse stimulation as well as 1 Hz stimulation (Manabe et al. 2012). Beside this, we observed that in a single myotube, EMG*stim* triggers action potentials and twitching as well as with 1 Hz ES, but at half of the stimulus intensity. Furthermore, even the percentage of muscle cells responsive to EMG*stim* given at low intensity was significantly higher.

We still do not know the precise mechanisms underlying the higher efficiency of EMG*stim* in triggering electrical and contractile activity in myotube effects. Nevertheless, we can speculate that the intrinsic variability present in EMG*stim* might induce small changes in muscle cell membrane potential, which facilitates cumulative depolarization. In turn, this allows even a subthreshold strength to

trigger action potentials that eventually transform weak stimuli into efficacious fatigue-resistant muscle activity.

Another interesting point is that canonical high strength electrical stimulation with stereotyped trains of pulses recruits motor units in a pattern that is non-selective, spatially fixed, and temporally synchronous and contributes to a greater muscle fatigue than voluntary actions (Bickel et al. 2011). Our results showed that stimulation with noisy electromyograms facilitates contractile activity throughout protocol delivery. This effect could be due to the fact that noisy stimulation prevents storage of muscular contraction inhibitors (Downey et al. 2014) or modifies ion channel biophysical properties, also through the activation of selected intracellular pathways (Zeng and Holmes 2010). Among the possible action mechanisms, noisy waves may also affect the cellular metabolism of muscle cells, similarly to what has been demonstrated at the spinal cord level with other protocols of asynchronous electrical stimulation (Wang et al. 2005).

The principal aim of the present work was "to establish the immediate useful contraction-inducing properties of the EMG*stim* pattern in relation to more conventional stimulation patterns rather than to examine the more longer-term myogenic and plastic changes induced by the stimulation protocols we used. Considering that prolonged 1 Hz stimulation contributes to muscle plasticity (Olson and Williams 2000; Kubis et al. 2003), we cannot exclude the possibility that prolonged EMG*stim* may better favor changes in contractile and metabolic properties in the muscle cells.

A detailed biochemical characterization of muscle cells after longer stimulation with EMG*stim* is therefore being planned for future experiments. Interestingly, an electrically-induced increase in extracellular ATP has been reported in skeletal muscle cultures (Buvinic et al. 2009) as well as the correlation between its content and the levels of reactive oxygen species (Sciancalepore et al. 2012).

Even if obtained in a simplified in vitro model of cultured muscular cells, our results can contribute to the knowledge of what occurs in vivo (Nikolić et al. 2012; Clausen 2013). ES patterns mimicking the firing of motoneurons and recruitment of healthy muscle fibers, also gave the best results in denervated muscles (Eberstein and Eberstein 1996). Nevertheless, beneficial effects have been observed in vivo with functional ES when it was performed by randomly modulating pulse frequency, amplitude and pulse-width in subjects with spinal cord injury (Thrasher et al. 2005).

In vivo, many factors influence the shape of the surface myoelectric signal in response to ES (*e.g.* distance between the electrodes and individual muscle fibers, muscle size, fiber orientation). Although the prediction of the effects of EMG*stim* in vivo is difficult because of the limits of the simplified cell model, we suggest that the effects of repetitive in vivo stimulation with noisy EMG patterns, might be more advantageous than conventional stimulations. It may prevent frequency-dependent conduction block (Robinson and Nielsen 1990; Watson et al. 2006; Noto et al. 2011) and/or induce the expression of nicotinic acetylcholine receptors within existing neuromuscular junctions and during remodeling of nerve-muscle contacts (O'Reilly et al. 2003) and, finally, it may favor satellite cell proliferation (Sciancalepore et al. 2012) associated with the expression of skeletal muscle genes (Juretic et al. 2007; Valdes et al. 2008). Moreover, since the density of current under the stimulating electrode is one of the main factors causing pain sensation, the efficacy of low stimulation with electromyographic patterns may be taken in consideration to reduce the discomfort associated with functional ES in vivo. This could represent a potential great advantage particularly when an intensive electrical stimulation is needed for the successful restorative effect (Kern et al. 2005).

Lastly, assuming the reliability of the in vitro cell model, ES performed on skeletal muscle cells could also represent a good alternative to the stimulation of the whole muscle, offering the advantage to explore the effect of ES at the single cell level and to identify the related molecular mechanisms, without interference from nerve released factors, hormones or blood flow variations.

In conclusion, the observation that EMG*stim* favors the excitability of a larger skeletal muscle cell population could represent a crucial step for clinicians in planning ES stimulation protocols.

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