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Biochimica et Biophysica Acta 1720 (2005) 117 – 124
BIOCHIMICA ET BIOPHYSICA ACTA
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Intrinsic ionic conductances mediate the spontaneous electrical activity of cultured mouse myotubes

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Received 7 September 2005; received in revised form 5 December 2005; accepted 6 December 2005
Available online 29 December 2005

Abstract

Mouse skeletal myotubes differentiated in vitro exhibited spontaneous contractions associated with electrical activity. The ionic conductances responsible for the origin and modulation of the spontaneous activity were examined using the whole-cell patch-clamp technique and measuring $[Ca^{2+}]_i$ transients with the Ca^{2+} indicator, fura 2-AM. Regular spontaneous activity was characterized by single TTX-sensitive action potentials, followed by transient increases in $[Ca^{2+}]_i$. Since the bath-application of Cd^{2+} (300 μM) or Ni^{2+} (50 μM) abolished the cell firing, T-type ($I_{Ca,T}$) and L-type ($I_{Ca,L}$) Ca^{2+} currents were investigated in spontaneously contracting myotubes. The low activation threshold (around −60 mV) and the high density of $I_{Ca,T}$ observed in contracting myotubes suggested that $I_{Ca,T}$ initiated action potential firing, by bringing cells to the firing threshold. The results also suggested that the activity of $I_{Ca,L}$ could sustain the $[Ca^{2+}]_i$ transients associated with the action potential, leading to the activation of apamin-sensitive SK-type Ca^{2+}-activated K^+ channels and the afterhyperpolarization (AHP) following single spikes. In conclusion, an interplay between voltage-dependent inward (Na^+ and Ca^{2+}) and outward (SK) conductances is proposed to mediate the spontaneous pacemaker activity in cultured muscle myotubes during the process of myogenesis.

Keywords: Skeletal muscle; Myotube; Spontaneous firing activity; T-type calcium current; L-type calcium current; SK K^+ current

1. Introduction

Skeletal muscle myotubes develop spontaneous changes in membrane potential [1–7] with accompanying contractions [2,3,5,6]. In mammalian skeletal myotubes differentiated in vitro, different discharge patterns and membrane potential oscillations were suggested to be coupled to transient changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [2,7,8]. The proportion of cells exhibiting spontaneous electrical activity and $[Ca^{2+}]_i$ transients increased with the duration of time that the cells were kept in differentiation media [7,8]. $[Ca^{2+}]_i$ variations were associated with mechanical activity only in the later phases of the in vitro myogenesis. More specifically, 3- to 5-day-old myotubes developed spontaneous electrical activity associated with $[Ca^{2+}]_i$ transients, but still uncoupled to detectable contractions, while in 6 day old myotubes, spontaneous activity and Ca^{2+} transients induced twitch contractions revealing a complete maturation of the excitation–contraction (E–C) machinery [9].

Although voltage-gated Na^+ channels are considered to be crucial for the origin of spontaneous electrical activity in myotubes [3,5,9,10], the results concerning their role are quite contradictory. Mammalian myotubes in vitro express both TTX-sensitive and TTX-insensitive Na^+ channels [5,10,11], but the TTX-sensitivity of spontaneous electrical activity is still not clear. In cultured rat myotubes, action potentials [3] and contractions [10] were found to be abolished by nanomolar concentrations of TTX, suggesting the involvement of TTX-sensitive Na^+ channels. In contrast, it was reported [5] that the functional Na^+ channels responsible for the spontaneous electrical activity in rat myotubes, were TTX resistant. Also, the role of voltage-gated Ca^{2+} channels is still debated. T-type Ca^{2+} currents ($I_{Ca,T}$), transiently expressed during the fusion of myoblasts into myotubes [4,12], were suggested to trigger the pacemaker-like activity of cultured rat myotubes, whereas a role

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doi:10.1016/j.bbamem.2005.12.001

in loading the intracellular Ca^{2+} stores was proposed for the L-type Ca^{2+} currents ($I_{Ca,L}$) [4]. On the other hand, since L-type Ca^{2+} channel antagonists silenced the spontaneous electrical activity [8,13] and the absence of α_{1S} dihydropyridine receptor (DHPR) expression was found to block their spontaneous Ca^{2+} oscillations, a role for $I_{Ca,L}$ in the initiation of the spontaneous activity has also been proposed [7]. Moreover, although Ca^{2+}-activated K^+ channels were found to mediate the skeletal muscle myotube afterhyperpolarization (AHP) [2] and small conductance Ca^{2+}-activated K^+ channels were found to be expressed in muscle depending on innervation [14], their role in the spontaneous electrical activity was not proved.

The contradictory results on the role of voltage-gated conductances in initiating the spontaneous activity could be due to the use of different experimental approaches; however, the coexistence of different spiking mechanisms in the same cell type must be also taken in account. In mouse myotubes, it has been recently demonstrated that the activity of the acetylcholine receptor channels is responsible for the "low frequency" activation of the excitation–contraction cascade (up to 0.45 Hz) [15]. Such a mechanism could overlap with a higher frequency firing due to the interplay between other intrinsic conductances. This is confirmed by the evidence that in the same cell model, cholinergic antagonists stop the spontaneous activity in the majority (about 70%) but not all myotubes [15]. Taking into account all these data, the detailed ionic mechanisms responsible for the initiation of spontaneous electrical activity during myogenesis remain so far unknown. The major aim of the present study was to study this physiological phenomenon in more detail. To do this, mouse myotubes spiking and contracting with the higher frequency pattern were investigated. The characterization of the different ionic conductances was examined using whole-cell patch clamp and videoimaging techniques. We propose that $I_{Ca,T}$ initiates the spontaneous "high frequency" pace-maker activity of the myotubes, and the interplay between TTX-sensitive voltage-gated Na^+, $I_{Ca,T}$ and SK Ca^{2+}-activated K^+ conductances sustains and determines the pattern of cell discharge.

A preliminary report of this work has already been presented [16].

2. Materials and methods

2.1. Cell culture

Cell cultures were established from mouse satellite cells, kindly provided by Prof. A. Wernig (University of Bonn). Briefly, cells were isolated from the hind-leg of 7-day-old male Balb/c mice killed by cervical dislocation as approved by local Animal Care Committee and in agreement with the European legislation. Muscle tissue was minced and then enzymatically dissociated with collagenase and trypsin [17]. Expansion and enrichment of desmin-positive cells was achieved by repeated plating and cultivation in Dulbecco's modified minimal essential medium (DMEM) supplemented with D-valine containing 20% fetal bovine serum. Myogenic cells (termed i28) could be maintained as exponentially growing myoblasts in the presence of a medium consisting of HAM'S F-10 containing 20% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). To induce cell fusion, the medium was replaced, 1 day after plating, with DMEM supplemented with only 2% horse serum and L-glutamine, penicillin, and streptomycin as above. The myoblasts were plated at a density of about 70,000 cells per 35 mm matrigel-coated dish. Cells were maintained at 37 °C in CO_2 (5%)-enriched air. Fusion media were renewed every 3 days to avoid loss of nutrients and growth factors. If not otherwise stated, the cells used in this study were aged between 5 and 9 days in fusion medium. Spontaneous contractile activity was determined in mouse myotubes by visual observation using an inverted microscope (Zeiss Axiovert 135, Oberkochen, Germany) at 400× magnification.

2.2. Monitoring contractile activity

Contractile activity was evaluated under bright field illumination at room temperature (22–24 °C), as the number of myotubes exhibiting twitches divided by the number of cells per optical field (×40 objective). Two different cell cultures were analyzed and at least 3 different dishes were observed for each cell culture. In more detail, 80 optical fields randomly chosen were observed.

2.3. Electrophysiological recording and data analysis

Both voltage and current data were obtained using the whole-cell patch-clamp technique [18]. To record the spontaneous electrical activity, access to the cytosolic compartment using the perforated patch-clamp method was preferred to conventional whole-cell recording, in order to provide exchange of small ions only and to avoid washout of intracellular second messengers. Thus, membrane potentials were recorded with amphotericin B-filled patch pipettes [19]. To reduce the action potential distortions, the fast current clamp mode was used [20]. All data were acquired at room temperature (22–24 °C) with 3–5 MΩ patch pipettes using an Axopatch 200B (Axon Instruments, Foster City, CA) amplifier, digitized through a Digidata 1321A interface (Axon Instruments) and stored on a PC-compatible hard disk. Currents were acquired at a sampling time of 200 μs and low-pass filtered at 2 kHz. For data acquisition and analysis, the pCLAMP software suite (v. 8.0, Axon Instruments) and Origin 7 (Microcal Software, Northampton, MA) software were routinely used.

Leakage and capacity currents were digitally subtracted using a P/4 protocol. A computer program was written to measure the parameters which characterized single action potentials: spike threshold was defined as the point at which the first derivative of voltage with respect to time exceeded 4 mV/ms; the amplitude was measured starting from the threshold value to the peak, and the width was measured at half-amplitude. Potentials were not corrected for the liquid junction potential, which was estimated to be +3 mV [21].

2.4. Simultaneous recordings of membrane potential and $[Ca^{2+}]_i$

The experiments were performed on myotubes plated onto matrigel-coated glass coverslips and loaded with the fluorescent Ca^{2+} indicator fura-2 pentacetoxymethylester (fura-2 AM). Records of membrane potentials and fluorescent signals were acquired with a conventional system driven by Imaging Workbench software (Axon Instruments). Cells were loaded by incubation (30 min, 37 °C) in NES solution supplemented with 10 mg/ml bovine serum albumin and 5 μM fura-AM. Myotubes were then bathed for 15 min at 37 °C in NES without dye to allow dye de-esterification. Loaded cells were then whole-cell patch clamped as described above. Cells were alternatively excited at 340 and 380 nm selected by a monochromator (Polychrome II T.I.L.L. Photonics GmbH, Martinszied, Germany). Fluorescence images were collected by a CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) at 25 frames/s. Recordings of membrane potential and fluorescence signals were synchronized and stored on a PC computer. Fluorescence ratio was calculated off-line [22]. The corresponding temporal plots, i.e., the variations in the mean value of fluorescence intensity, were calculated from ratio images in the areas of interest.

2.5. Statistical analysis

All data are expressed as the mean±S.D. with n being the number of cells tested, and statistical significance was assessed using the Student's unpaired t test at the $P<0.05$ level. Membrane capacitance was determined by integrating the area of initial transient membrane responses to a 5-mV hyperpolarizing command.

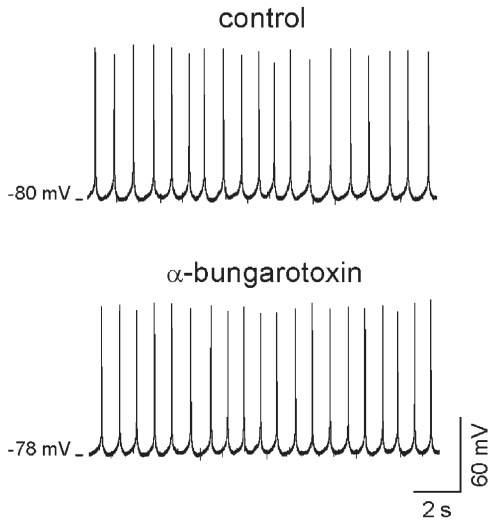

Fig. 1. The nicotinic acetylcholine receptor blocker α-bungarotoxin, does not affect the myotube firing rate. Representative records of spontaneous action potentials recorded before and after 15 min application of α-bungarotoxin (10 μM) to a contracting myotube after 7 days in differentiation medium.

2.6. Solutions and drugs

Simultaneous recordings of membrane potential and $[Ca^{2+}]_i$ as well as the recordings of spontaneous electrical activity were performed in normal external solution (NES) containing (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 were backfilled with the same solution containing amphotericin B (150 μg ml^{-1}) made fresh from a stock solution (20 mg ml^{-1} in DMSO) kept at 4 °C. Na^+ currents were recorded in a bath solution containing (in mM): 100 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH = 7.3. Tetraethylammonium chloride (TEA, 40 mM) was added to the bathing medium to minimize outward K^+ currents and 0.5 mM $CdCl_2$ to block Ca^{2+} currents. Patch pipettes were filled with the following solution (in mM): 120 CsCl , 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES. Ca^{2+} currents were recorded in a bath solution containing (in mM): 135 TEACl, 2.5 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 5.6 glucose. Patch pipettes were filled with the following solution (in mM): 130 CsCl, 0.005 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 10 HEPES, 1 EGTA, 2 MgATP. Drugs were bath-applied by a gravity-driven perfusion system.

2.7. Chemicals

Dulbecco modified Eagle's medium, horse serum, antibiotics and L-glutamine were purchased from ICN Biomedicals (Costa Mesa, CA, USA); bovine foetal calf serum from PAA Laboratories (Linz, Austria); amphotericin B and TTX were purchased from Tocris, Cookson Ltd., UK. All other chemicals were from Sigma (St. Louis, MO, USA).

3. Results

Spontaneous action potentials associated with twitches were detected in muscle cell cultures starting from the third day in differentiation medium, after fusion of myoblasts into multinucleated myotubes. The percentage of contracting myotubes progressively increased, reaching a peak around day 7, when the mean percentage of active cells was 37.2 ± 20.4% (total number of cells = 449) [see also 2,15]. To investigate the voltage-dependent conductances triggering the spontaneous events, experiments were performed on cells exhibiting firing and twitching in the "high frequency" mode. To systematically analyze such firing, only myotubes showing long-lasting spontaneous activity were considered.

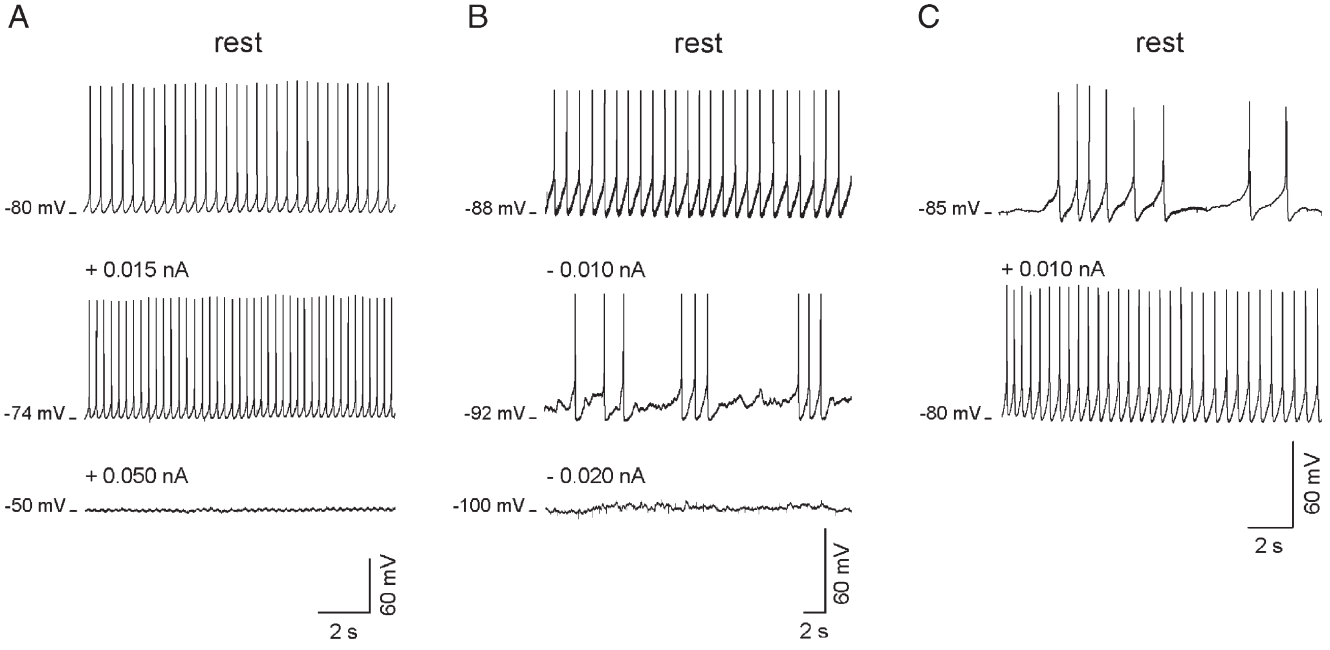

Fig. 2. Representative records of spontaneous action potentials recorded in current clamp in contracting myotubes after 7 days in differentiation medium. (A) The firing pattern is shown at resting potential (rest) and during steady injection (+0.010 nA and +0.050 nA) of positive DC current. (B) The myotube firing is shown at resting potential and after injection of negative DC current. (C) The irregular firing observed in a myotube at rest could be converted to a regular one by injecting depolarizing current.

3.1. Voltage-dependency of the "high frequency" spontaneous firing

A sustained tonic firing of single spontaneously-occurring overshooting action potentials characterized the electrical membrane activity of mouse myotubes. The mean frequency of spontaneous activity was 1.64±0.15 Hz, with a coefficient of variation (CV) of 0.24±0.04 (n=28). Regular firing was typically observed (Fig. 1, control), sometimes interrupted by silent periods; rarely, even bursting activity was noted. The mean action potential amplitude was 78±17 mV, with half-width=7.7±5.8 ms and threshold=−44±9.3 mV (n=19). An AHP followed each single action potential. Bath perfusion of the nicotinic receptor antagonist α-bungarotoxin (10 μM) for 10–20 min did not significantly change the frequency of spike discharge (1.52±0.24 Hz versus 1.83±0.45 Hz, n=4; Fig. 1); thus, such activity was independent from the activation of the acetylcholine receptor channels sustaining the previously characterized "low frequency" pattern [15]. Due to membrane potential oscillations, it was not possible to accurately measure the myotube resting potential; however, in the cells that occasionally stopped firing, it ranged from around −60 mV to −45 mV.

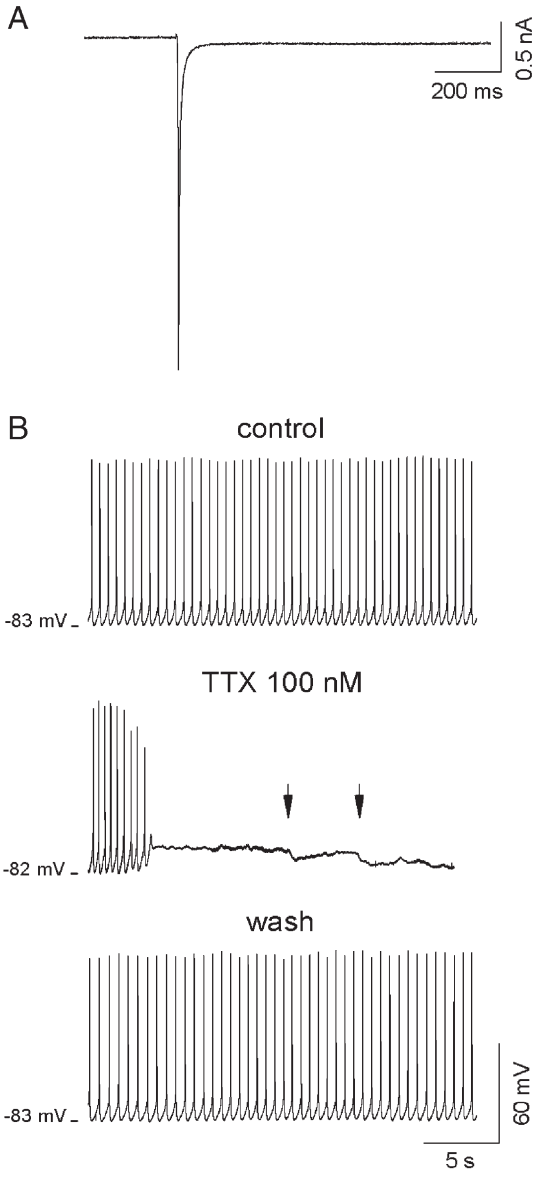

Fig. 3. Na^+ currents mediate the single action potentials. (A) Representative trace recorded under voltage clamp in a myotube. Na^+ current is elicited by a voltage step from a holding potential of −90 mV to −40 mV (average of 3 records is shown). Capacity and leakage currents have been subtracted as described in Materials and methods. (B) Single action potentials recorded in perforated patches were completely blocked in the presence of TTX (100 nM) which brings the membrane potential to positive values. Injection of hyperpolarizing currents (arrows) returns the cell to negative membrane potentials, where the activity is still silent. Washout of TTX (15 min) restores the original spontaneous firing activity.

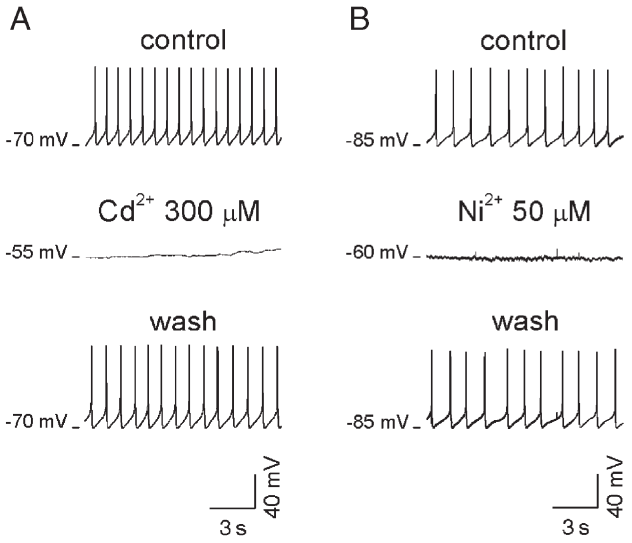

Fig. 4. Spontaneous electrical activity is silenced by Ca^{2+} channel blockers acting on L- and T-type Ca^{2+} currents. Spontaneous action potentials recorded at control level (I=0) before and after application of 300 μM Cd^{2+} (A) or 50 μM Ni^{2+} (B). Maximum negative potential is reported. Note the shift of membrane potential in a depolarizing direction after Cd^{2+} or Ni^{2+} application.

Spontaneous activity was highly dominated by the membrane potential values. Thus, injection of steady depolarizing (Fig. 2A) or hyperpolarizing currents (Fig. 2B) could change the spontaneous firing pattern in a consistent manner suggesting that voltage-dependent intrinsic ionic conductances could be responsible for this behaviour. Injection of a small depolarizing current increased the firing rate, whereas a higher depolarizing current (bringing the potential to approximately −50 mV), converted the tonic firing cell into a silent one (Fig. 2A). The injection of hyperpolarizing current made the activity irregular, decreasing the firing rate until a complete stop was seen (Fig. 2B). Interestingly, the regularity of the spike discharge was also a clear function of the membrane potential value. Myotubes, with irregular activity at rest, could be artificially brought to regular firing by injection of steady depolarizing current (Fig. 2C).

3.2. Na^+ currents

To investigate Na^+ currents, voltage clamp recordings were performed in whole-cell configuration in contracting myotubes, silenced by superfusion of TEA and $CdCl_2$-containing bathing

medium. Na^+ currents were regularly recorded after subtracting the voltage-insensitive "leak" currents. In all cells, membrane depolarization activated a transient Na^+ inward current with a mean activation threshold of −53±6.1 mV (n=30). A typical record is shown in Fig. 3A where the Na^+ current was elicited by a voltage step to −40 mV from a holding potential of −90 mV. To examine the contribution of Na^+ channels in driving the spontaneous activity, we studied the effect of Na^+ channel blockers on the firing discharge. Application of TTX (100–300 nM) reversibly suppressed the spontaneous cell firing (n=13) bringing the membrane potential to a plateau (range −65 mV to −50 mV), negative to the threshold value (Fig. 3B). Similar values of membrane potential were occasionally reached spontaneously when the cell stopped firing. In this condition, even the artificial injection of hyperpolarizing current (see arrows in Fig. 3B) did not induce spontaneous activity. This suggest that TTX-sensitive Na^+ channels are responsible for the action potential initiation at the spike threshold.

3.3. Ca^{2+} currents

The role of Ca^{2+} channels in generating spontaneous electrical activity was also pharmacologically investigated. In particular, the non-specific Ca^{2+} channel blocker Cd^{2+} (300–500 μM, n=3), or the T-type Ca^{2+} channel antagonist Ni^{2+} (50 μM, n=4) were added to the bathing medium. In all the cells tested, inhibition of membrane Ca^{2+} channels reversibly stopped the spontaneous electrical activity, setting the membrane potential at a depolarized plateau level of around −60 to −50 mV (Fig. 4A, B). The activity continued to be absent even after injecting hyperpolarizing current to bring the membrane potential to a more negative level (data not shown).

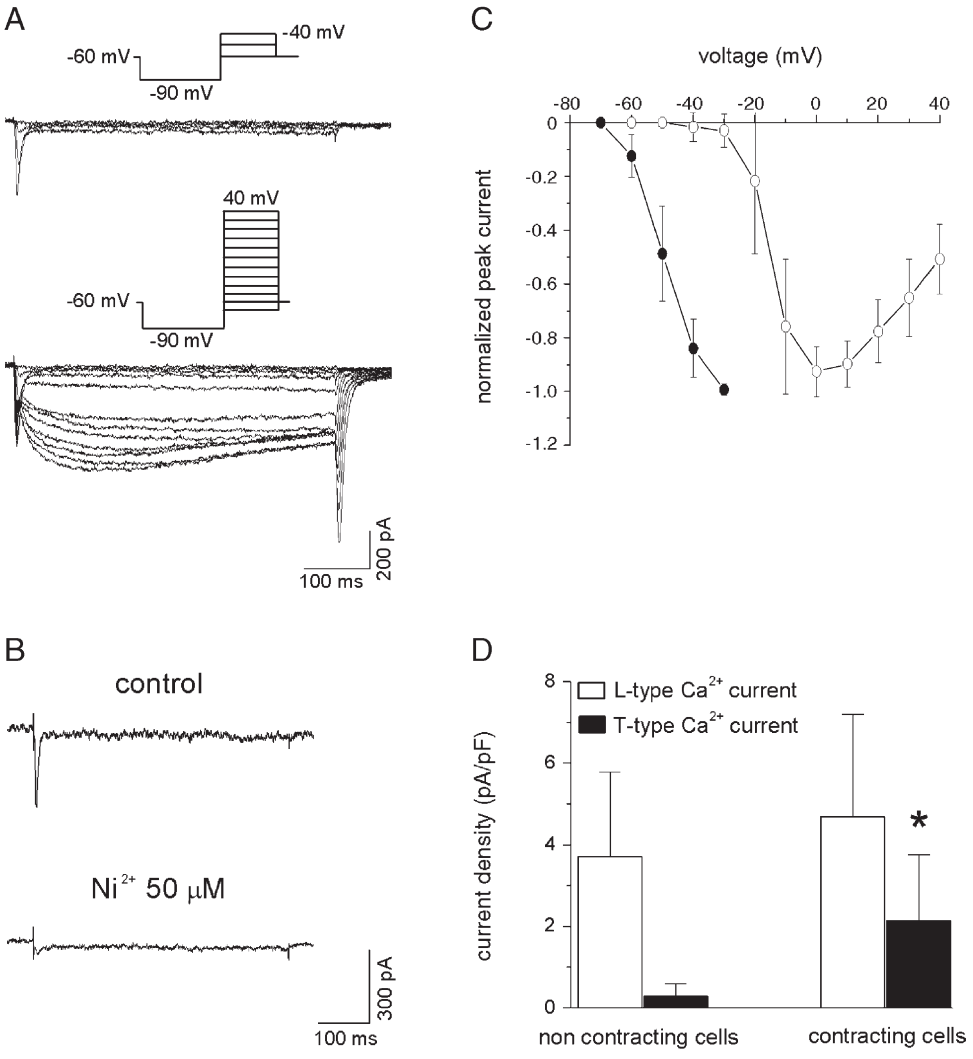

Fig. 5. Representative T- and L-type Ca^{2+} currents elicited in myotubes. Voltage protocols were designed to isolate $I_{Ca,T}$ and $I_{Ca,L}$ currents, respectively. (A) T-type (top) and L-type (bottom) Ca^{2+} currents are elicited by the activation pulse protocols shown in the insets. Capacity and leakage currents have been subtracted as described in Materials and methods. (B) Representative example of $I_{Ca,T}$ recorded at −40 mV is reduced by 50 μM Ni^{2+}. (C) Mean peak $I_{Ca,T}$ (filled circles, n=10) and $I_{Ca,L}$ (open circles, n=10), normalized to their maximum amplitude values (at −30 mV and +0 mV, respectively) are plotted as a function of the membrane potential. (D) The mean density±S.D. of $I_{Ca,T}$ and $I_{Ca,L}$ (peak current values at −40 mV and at +10 mV, respectively normalized to the corresponding effective membrane capacitance) is estimated for non-contracting (n=9) and contracting (n=11) cells. The asterisk indicates statistical significance (P<0.05).

In contracting myotubes, silenced by addition of extracellular TEA in a Na^+-free solution, voltage-clamp experiments invariably revealed the presence of both $I_{Ca,T}$ and $I_{Ca,L}$ ($n=20$). A depolarization of the membrane potential from −60 mV to −40 mV (preceded by a hyperpolarizing pulse to −90 mV), elicited only rapidly inactivating T-type Ca^{2+} currents (activation threshold −60 mV) (Fig. 5A, top); further depolarization activated the L-type Ca^{2+} current (Fig. 5A, bottom). In all the examined cases ($n=4$), the fast-inactivating Ca^{2+} current was reduced (72±29%) in the presence of Ni^{2+} as would be expected of $I_{Ca,T}$ (Fig. 5B). Comparison of the current–voltage relationship for $I_{Ca,T}$ and $I_{Ca,L}$ (Fig. 5C) shows that $I_{Ca,T}$ (filled circles) was activated at more hyperpolarizing potentials than $I_{Ca,L}$ (open circles). In the same cells, when the holding potential of −60 mV was followed by a pre-pulse of −30 mV, $I_{Ca,T}$ was not detected because it was inactivated, whereas $I_{Ca,L}$ started to appear at around −20 mV (mean = −22±5 mV, $n=19$). It is interesting to note that while $I_{Ca,L}$ was recorded in both contracting and not-contracting myotubes, $I_{Ca,T}$ was always present in contracting ($n=20$) but absent ($n=4$) or clearly reduced ($n=5$) in non-contracting cells. In more detail, the average peak amplitude of the $I_{Ca,T}$ was 279±200 pA at Vm −50 mV and 404±211 pA at Vm −40 mV in contracting ($n=23$), while it was 42±18 pA at Vm −50 mV and 101±40 pA at Vm −40 mV when detected in non-contracting cells ($n=7$). The calculated current density (pA/pF) of $I_{Ca,T}$ at Vm −40 mV was 2.1±1.6 ($n=11$) and 0.3±0.3 pA/pF ($n=9$) for contracting and non-contracting cells, respectively. A comparison between $I_{Ca,T}$ and $I_{Ca,L}$ mean density is shown in Fig. 5D.

The low-voltage activation of $I_{Ca,T}$ and the association between the presence of functional $I_{Ca,T}$ and the occurrence of spontaneous electrical activity, strongly suggest the role of $I_{Ca,T}$ in depolarizing the membrane potential to the threshold for firing initiation.

3.4. K^+ currents

Simultaneous recordings of electrical membrane activity and $[Ca^{2+}]_i$ variations carried out in "high frequency" spiking myotubes, revealed that a $[Ca^{2+}]_i$ transient follows each single action potential (Fig. 6A). The mean delay between the peaks of action potentials and Ca^{2+} transients was 141±32 ms ($n=5$). Since such increase in $[Ca^{2+}]_i$ could activate Ca^{2+}-dependent K^+ conductances, the putative role of the small-conductance Ca^{2+}-activated (SK) potassium currents in modulating spontaneous electrical activity was examined pharmacologically by comparing such activity in control and in the presence of the SK channel blocker apamin. A clear increase of firing frequency (from 1.83±0.2 Hz to 2.61±0.34 Hz, $n=7$, $P<0.05$) was commonly observed after 2–3 min exposure to apamin (100 nM) (Fig. 6B). Apamin application did not affect the action potential threshold (from 46±7 mV to 44±5 mV, $n=7$), or the amplitude (from 93±18 to 93±14, $n=7$), whereas it shortened the AHP duration. As an example in Fig. 6B, the AHP duration, measured at −60 mV was 10.35±1.9 ms in control and 6.07±0.9 ms after apamin application ($n=21$ spikes). The short AHP that remained in the presence of apamin, was most likely due to the activity of voltage-sensitive fast K^+ conductances [2]. Occasionally, the spontaneous activity was completely blocked ($n=2$, Fig. 6C). Thus, our data suggest that SK-type Ca^{2+}-dependent K^+ currents mediate the AHP regulating the interval between the spikes.

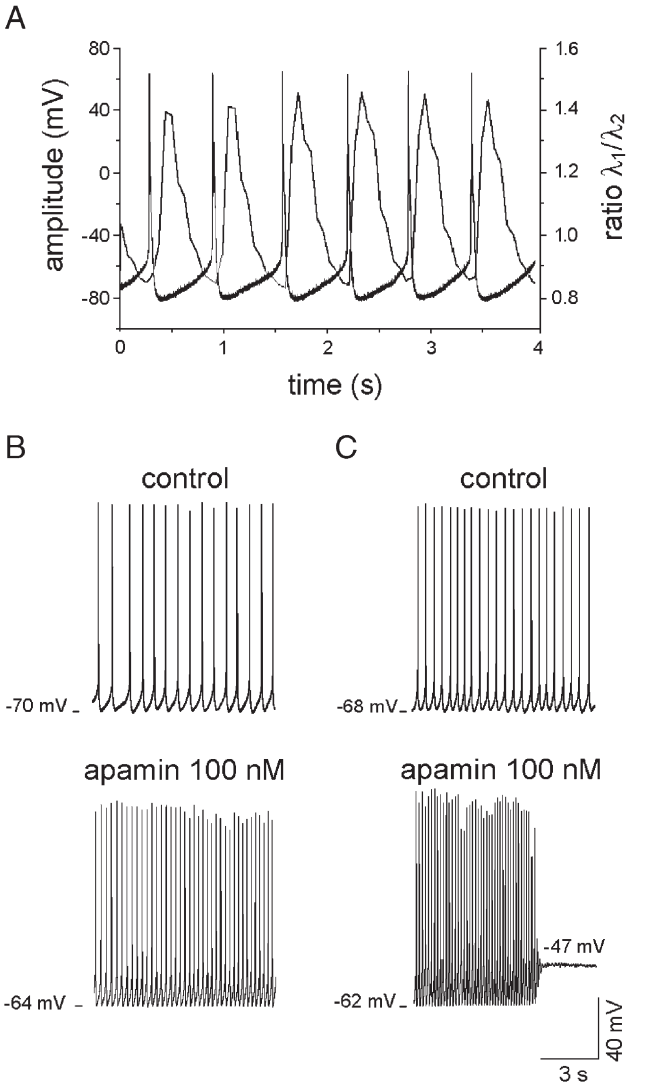

Fig. 6. Spontaneous action potentials, followed by $[Ca^{2+}]_i$ transients activate an apamin-sensitive AHP. (A) Spontaneous action potentials and transient increases in $[Ca^{2+}]_i$ simultaneously recorded in a mouse myotube in culture. Membrane potentials were recorded at 'resting' level ($I=0$) in perforated patches and $[Ca^{2+}]_i$ changes were detected by videoimaging from a single contracting myotube. Note that spontaneous action potentials precede the Ca^{2+} transients. (B) The frequency of the spontaneous activity is modulated by SK K^+ currents. Representative traces show the electrical spontaneous activity in control and after 100 nM apamin application; note the increase in firing rate. (C) Occasionally, after apamin addition at the same concentration, the firing rate increased dramatically, completely blocking the AHP and eventually abolishing the spontaneous activity.

4. Discussion

This article reports a study of the ionic conductances responsible for the spontaneous "high frequency" firing of cultured mouse myotubes. We provide evidence that the

membrane potential critically affects the frequency of spontaneous action potentials and that inward and outward conductances initiate and modulate the firing properties.

More specifically, low-threshold activated T-type Ca^{2+} channels, de-inactivated by the hyperpolarization induced by the apamin-sensitive AHP, are suggested to bring the membrane potential positive to the threshold for TTX-sensitive spike firing.

4.1. Contribution of Na^+ and Ca^{2+} channels

The activation threshold (around −55 mV) of the voltage-gated Na^+ current suggests the role of such channels in the membrane depolarization leading to firing initiation. The involvement of TTX-sensitive Na^+ channels in the generation of spontaneous action potentials was confirmed by the reversible blocking effect of 100 nM TTX on the spontaneous pacemaker activity. This is in accord with the data reported in rat skeletal myotubes, where TTX abolished the spontaneous electrical activity with an EC_{50} of 12 nM [3] and the spontaneous contractile activity with a K_i of 26 nM [10]. However, our results suggest that spontaneous firing requires not only TTX-sensitive Na^+ currents but also $I_{Ca,T}$. So far, $I_{Ca,T}$ was found to have a critical role in increasing the $[Ca^{2+}]_i$ required for myoblast fusion, a process essential to skeletal muscle development and repair [23], whereas its role in governing the spontaneous electrical activity of myotubes was not completely elucidated [4]. The block of the spontaneous activity by Ni^{2+} and the low threshold activation of such Ca^{2+} currents suggest a role of $I_{Ca,T}$ in the initiation of spontaneous electrical activity. Accordingly, we observed that only myotubes characterized by higher $I_{Ca,T}$ density were firing and contracting.

4.2. Role of Ca^{2+}-activated K^+ channels

Electrophysiological and Ca^{2+} imaging experiments, performed simultaneously, revealed that Ca^{2+} transients follow the action potentials, suggesting that single spike-generated Ca^{2+} dynamics could elicit SK currents. The change in frequency of spontaneous activity induced by the specific SK channel blocker apamin, indicates that SK conductances change the AHP duration and thereby regulate the interspike interval [2,24]. Therefore, intrinsic properties of cultured myotubes such as the density of functional SK channels and their interaction with $I_{Ca,T}$ may therefore critically contribute to the spontaneous cell firing by modulating its rate. In line with this, SK channels are expressed in denervated muscle [14] or in muscles of myotonic dystrophy-affected patients [25]; in both conditions, trains of action potentials are observed, confirming the role of such conductances in controlling the cell excitability.

In agreement with previous results showing that the absence of the α_{1S} subunit of L-type Ca^{2+} channels abolished the spontaneous Ca^{2+} transients [7] and that blockers of L-type Ca^{2+} channels silenced the spontaneous activity in mouse myotubes [8], we also found that L-type Ca^{2+} channels are essential in determining myotube firing and twitching. It must be taken into account that in skeletal muscle, the excitation–contraction coupling is based on the mechanical coupling between the L-type Ca^{2+} channels and ryanodine receptors. Due to this, the activation of L-type Ca^{2+} channels upon membrane depolarization causes the opening of ryanodine receptors and Ca^{2+} release from the sarcoplasmic reticulum [26]. Therefore, we suggest that during the firing, the L-type Ca^{2+} channels could provide SK channels with an efficient Ca^{2+} signal amplified by the Ca^{2+} release from intracellular stores.

In conclusion, we provide evidence that the "high frequency" spontaneous electrical activity in mouse myotubes results mainly from an interplay between Na^+, Ca^{2+} and Ca^{2+}-activated K^+ currents. This mechanism therefore appears different from that controlling the "low frequency" firing, where the autocrine activation of acetylcholine receptor channels plays a major role. The identification of the two firing mechanisms and their different origin might represent a new basis for further examination of the effects of different patterns of spontaneous electrical activity on the maturation of skeletal muscle cells during the process of myogenesis.

Acknowledgements

The project was supported by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca and the Regione Friuli Venezia Giulia. The authors are grateful to Drs. A. Constanti (School of Pharmacy, University of London) and E. Cherubini (S.I.S.S.A., Trieste) for useful comments on the manuscript.

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