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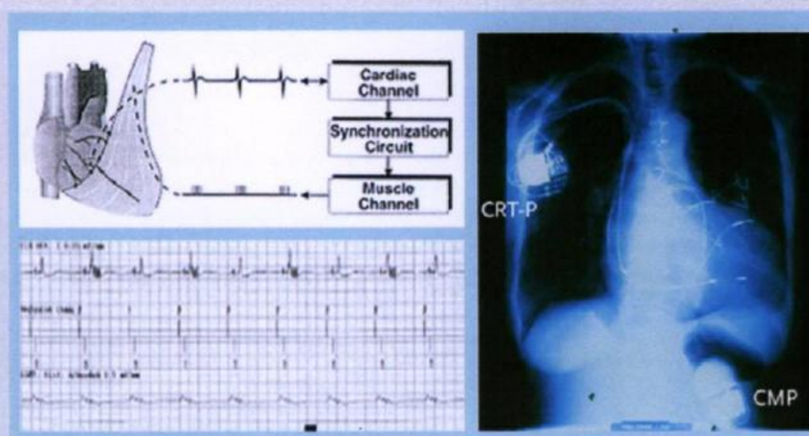

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Biophysical effects of high frequency electrical field (4-64 MHz) on muscle fibers in culture

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Abstract

Effects of oscillating electrical fields on living cells depend on the frequency. Electrical field oscillating in the range of MHz can induce cell membrane deformation which may end in cell damage or stimulation. The issue has been studied in red blood cells, but never in excitable cells. In this study we investigated the behaviour of murine muscles and single muscle fibers exposed in vitro to an oscillating electrical field in the MHz range. The commercial set-up Rexonage ™ (Telea srl), providing a particular frequency spectrum in the range 4-64MHz that is patented as "quantum molecular resonance stimulation", was used as a generator of electrical field and a wide range of powers was examined. Muscles or muscle fibers were placed on the bottom of Petri dishes and electrical field was applied between a needle electrode immersed in the medium and movable by means of a micromanipulator and an electrode placed under the bottom of the dish. While high power stimulation produced a fast and well localized cut of the fibers, low power stimulation caused a reversible deformation of the membrane. Such deformation was accompanied by a membrane depolarization and an increase of cytosolic free calcium, which were detected with fluorescent probes. Both the changes of membrane potential and the variations of free calcium concentration strictly followed the time course of electrical field application and removal. In conclusion, the present results demonstrate that excitable cells, such as muscle fibers, respond to the application of high frequency electrical field even when the threshold for action potential is not reached. This might lead to the activation of intracellular signalling pathways even without contraction.

Key Words: High frequency electrical field, membrane potential, cytosolic calcium, muscle fibers

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The behaviour of biological materials exposed to electromagnetic fields has become a growing research area in the last years. The interest is stimulated by the variety of frequency-dependent phenomena occurring in the biological tissues that are commonly explained from a theoretical point of view in terms of dielectric dispersion models [7]. The studies are mainly focused on the characterization of the responses of different cell kinds [7, 12] and to possible implications in diagnostic and applications to cell-manipulation [16].

The dispersion phenomena are commonly classified as α-dispersion, β-dispersion and γ-dispersion depending on the corresponding frequency spectrum of the underlying physical processes [1]. The α-dispersion is related to dielectric relaxation of free water and occurs in the range of GHz. The β-dispersion occurs in frequency region between 10 kHz and 100 MHz and is well explained by structural polarization phenomena or the so-called Maxwell-Wagner effect, involving the cytoplasm, the plasma membrane and the surrounding extracellular medium [8]. The γ-dispersion occurs in the range 100 Hz-10 kHz and is related with the variation of the cell membrane capacitance.

Taking advantage from the effects induced by the alternating field stimulation [11], such as the dipole generation leading to cell displacement and to cell deformation, a number of investigations have been carried out on the structural characterization of the cell components [10]. The viscoelastic properties of the cell membrane have been studied in erythrocytes immersed in an oscillating electrical field [5, 6] and applications of these phenomena were developed in order to fabricate devices for cell screening based on dielectrophoretic mechanisms [2, 13]. Some other

studies have considered AC (Alternating Current) stimulation as a mean to break the membrane structure in electrofusion and cell poration experiments not only from the point of view of the dielectrophoretic deformation [15] but also from the point of view of the transmembrane potential variations induced during AC field application [14].

In the present report, we focused mainly onto the effects induced by non uniform low power AC stimulation with high (MHz) frequencies on a specific kind of biological models: muscles and muscle fibers. Taking into account the excitability of these cells, the experiments have examined the physiological effects of the AC stimulation in terms of fluctuations of the trans-membrane potential and alterations of cytoplasmic calcium concentration. The high frequency stimulation was also directly compared on the same preparations with the classical bipolar field stimulation obtained with two platinum electrodes and able to elicit action potentials, calcium transients and contractions.

Materials and Methods

Muscle Tissues and Cells Preparation Protocol

Experiments were carried out on murine adult muscle fibers enzymatically dissociated and kept in culture as described by [4]. Mice were killed by cervical dislocation and Flexor Digitorum Brevis (FDB) muscle was dissected out and placed in Tyrode solution (NaCl 140 mM; KCl 2 mM; CaCl2 0.5 mM; MgCl2 2 mM; HEPES 10 mM; glucose 5 mM) containing 0.2% type I collagenase and 10% fetal bovine serum (FBS) for 1 hour at 4°C and then for 1 hour at 37°C. After several washes in Tyrode solution containing 10% FBS to block the collagenase effect and stabilize the fibers, the muscle was gently dissociated through a Pasteur pipette with a large opening in a glass falcon test tube to obtain single fibers. Isolated fibers were plated on cover slips covered with mouse laminin, which produced fiber attachment within 1 hour. Fibers were maintained in Tyrode solution supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator, with 5% CO2 at 36.5 °C and used for experiments on the second day after dissociation.

Few experiments were carried on whole murine Extensor Digitorum Longus (EDL) muscles freshly dissected from the leg and immediately pinned at both ends on the bottom covered with Silgard of a Petri dish filled with oxygenated Krebs solution.

The use of the animals and the experimental protocol was approved by the Department Ethical Committee. All efforts were made to minimize animal suffering and to use only the number of animals strictly necessary to obtain reliable data.

Experimental Setup

The experimental setup was designed to generate the oscillating electric field around single muscle fibers or EDL muscles placed inside a Petri dish. A commercial stimulator (Rexonage™, Telea), representing the edge technology applied in many surgical and medical tasks, was adopted because of its own peculiar range of stimulation frequency. In fact the stimulator output signal is a periodic sinusoidal signal with a particular harmonic spectrum in the range between 4 and 64 MHz as patented by TELEA s.r.l. as “quantum molecular resonance stimulation”. The amplitude ranges from few Volts to about 1 kVolts depending on the power setting. A detailed characterization of the output signal amplitude as function of the power settings was performed in order to evaluate the electric field intensity induced in the extracellular environment. The stimulation protocol envisaged the application of the electric field between a Pt microelectrode of 65 μm diameter, immersed in the perfusing medium very close (<100μm) to the membrane surface, and the bottom side of the culture dish in contact with a ground reference plate in order to close the current pathway. The microelectrode holder was mounted on a 3-axis stepper manipulator stage which allowed the fine control the positioning of the electrode tip in relation to the cell membrane with the help of a stereo-microscope. A simulation of the spatial properties of the stimulation field, generated with the electrode dipped in the perfusion medium (with known dielectric constant and conductivity parameters) at different distances and tilting angles with the muscle fiber surface, was developed. The simulation is shown in Figure 1 and allows the evaluation of the concentration of the electric field and its maximum spatial extension.

Optical Microscopy

A custom upright stereo-microscope from a Mytutoyo stage with very long working distance objectives (10X, 20X and 50X) was used with visible light illumination either for imaging selected regions of isolated muscle or single FDB fibers before, during and after stimulation sessions. By means of a monochromatic camera (SONY-CCD) and video capturing DAQ board installed in a PC, movies of the cell fibers were recorded and digitized. Imaging data analysis was carried out by means of ImageJ software.

Fluorescent Probes and Microscopy

The effects of the stimulation on physiological parameters, cytosolic calcium concentration and membrane potential, were studied by means of fluorescent dyes that change their fluorescence intensity in response to calcium ion binding or to a change in membrane potential. For cytosolic calcium imaging, cell permeant AM ester Fluo4 (F14201, Molecular Probes) was diluted with a concentration of 3μM into the cell loading buffer (125mM NaCl, 5mM KCl, 1mM MgSO4, 1mM KH2PO4, 5,5mM glucose, 1mM CaCl2, 20mM HEPES and 1% BSA). After 30 min loading phase at 37° C, cells were incubated in loading solution

without BSA and Fluo4 for 10 min at 37°C and then the cover-glass was mounted in the imaging chamber. To record transmembrane potential variations, FDB fibers were immersed for 60 min in 4μM Di-8-ANEPPS (D3167, Molecular Probes) dissolved in the loading buffer in order to obtain a homogeneous binding to the cell membrane. The fluorescence imaging setup was composed by a Nikon TE2000 inverted research microscope with a 60X APO TIRF NA1.45 oil immersion objective and a 20X NA 0.8 dry objective. All the imaging data were acquired using a Hamamatsu ImageM back-tinned multiplying camera controlled by the Nikon NIS Advanced Research software and by the Hamamatsu proprietary software Wasabi. The fluorescence filter configuration for Di-8-ANEPPS imaging was based on a 488/30nm excitation filter, a 505nm dicroic mirror and a 590nm long-pass emission filter; for Fluo4 was similar except for the emission filter which was replaced with a 535/50nm. The experimental protocol aimed to compare in the same cell the membrane potential changes/calcium transients obtained with high frequency stimulation electrode with the responses obtained with normal field stimulation with supramaximal 30V square bipolar single stimuli.

Results

Simulation of the High Frequency Electrical Field

The spatial distribution of the high frequency (MHz) electrical field of stimulation in a physical system composed by a Pt electrode, an extracellular medium and a perspex substrate was studied with a computer simulation. Simulation, calculated for stimulation frequency of 4MHz, showed that, because of the particular shape and dimensions of the electrode, the volume interested by the stimulation was very confined (Figure 1).

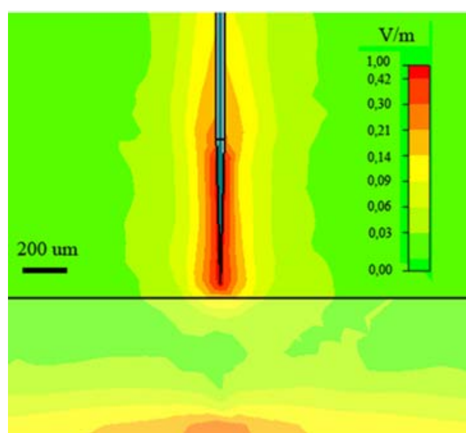

Fig. 1 *Spatial distribution of the high frequency electrical field. The black horizontal line represents the bottom of the petri dish where a second plate electrode is placed. The magnitude of the field is represented by means of a normalized pseudocolor scale.*

At an average distance of 80 μm from the electrode, the absolute value of the induced electric field was about 30% of its maximum value and decrease to 6% at a distance from the tip of 200 μm. Beside the confinement, simulation predicted a very high degree of polarization of the electric field, which is mainly directed along the electrode longitudinal axis (Figure 1). Actually, the transversal components reached at their maximum only the 40% of the longitudinal values, also when the tilting angle was reduced from 90° to lower values.

Effects of MHz Electrical Field on Muscle Fibers in Relation to Power Applied

Taking into account the shape and the polarization of the electrical field predicted by the simulation and exploiting the fine control of the electrode positioning allowed by the micromanipulator, the electrode tip was placed at optimal distance from the cell membrane.

During low power experiments, the tip was located at about 50 μm from the membrane surface, while in higher power experiments, conducted to induce cell permanent morphological changes, the tip was placed even more close to the cell membrane. The power settings corresponded to applied peak-to-peak voltage amplitude in the range of 500V-800V in the high power configuration and in the range 100V-400V in the low power one. The effects on the muscle cells caused by high power and low power application of the electrical field (thereafter indicated as stimulation) were very different and are shown in Figures 2-4.

High power stimulation with the electrode perpendicularly placed 100 μm apart from the membrane caused disorganization in the muscle scaffold with permanent changes in shape and position of the fibers previously aligned along the longitudinal muscle axis. If the tip was more close to the membrane, muscle fibers were transversally cut in a position corresponding to the electrode tip with a background particle chaotic movement (Figure 2).

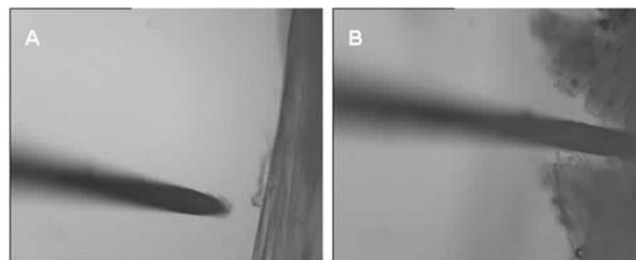

Fig. 2 *Cutting effects of high power electrical field on muscle fibers. Panel A shows the electrode placed perpendicular to the muscle surface before electrical field is applied. Panel B shows the cut produced by high frequency high power electrical field. Note the sharp cut of individual fibers.*

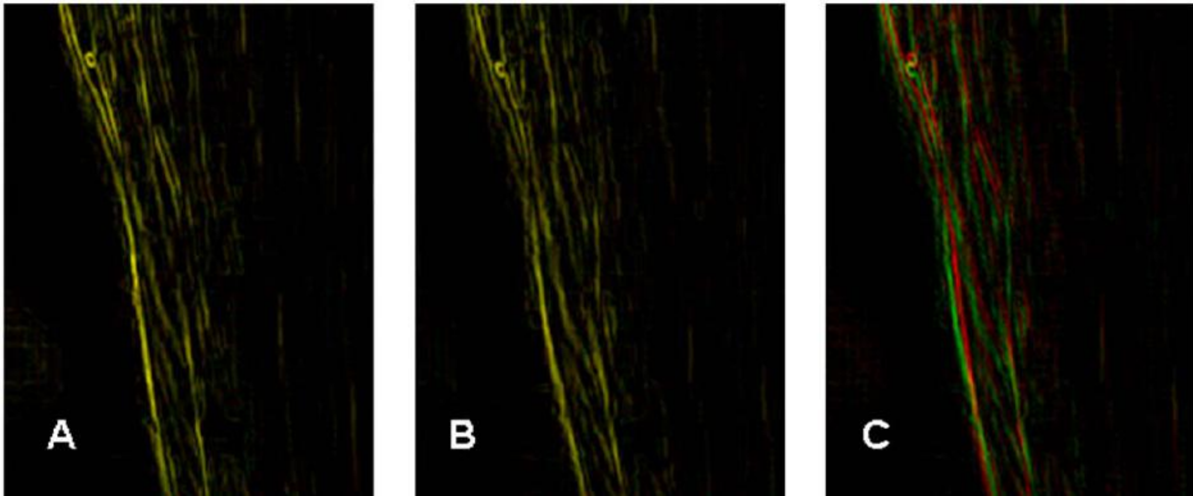

Fig. 3 *Changes in muscle fiber shape induced by low power electrical field . A) detail of isolated EDL muscle close to the electrode before stimulation, B) the same region during stimulation, C) merge of the two images : A in red and B in green. Changes in shape of individual fibers are detectable by means after the processing of the images with a "find edge algotithm".*

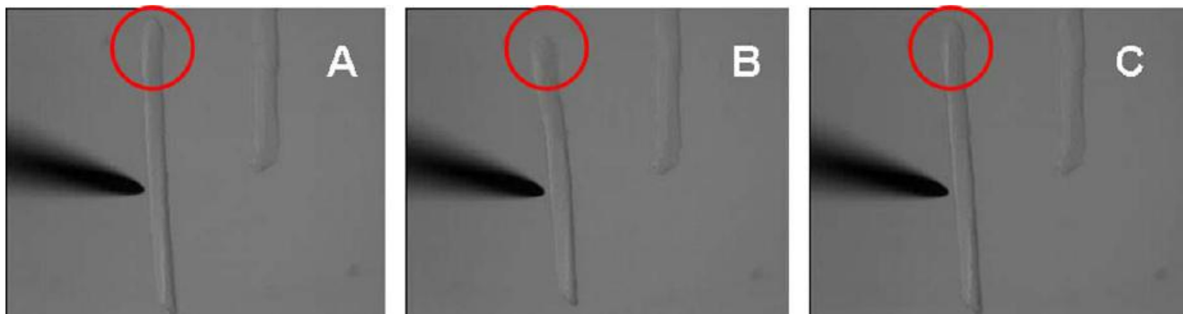

Fig. 4 *Slow contractile response induced by electric field in a single muscle fiber. A) before stimulation, B) during stimulation, C) after stimulation. The electrical field was applied for 500 ms (stimulation).*

Fig. 5 *Migration of particles inside the electrical field during stimulation. A group of three particles are visible close to the tip of the electrode on the left side and a single fiber on the right side. The relative changes in position are detectable by eye comparing the three images taken at subsequent times.*

The damage in the fiber membrane morphology was restricted to a small (about 150 µm diameter) region approximately centered around the tip of the electrode, thus corresponding to the predicted size of the electrical field (compare with Figure 1). The surrounding regions appeared almost completely unaffected, thus confirming the extraordinarily confined cut effect.

The low power setting with the tip 50 µm apart the muscle surface induced on the muscles and muscle fibers transient effects that completely disappeared after the end of the stimulation phase. During stimulation, muscle fibers showed a movement reminiscent of a slow contraction (Figure 4). Also in this case, the response was restricted to the limited region of the muscle more close to the electrode tip,

and it was characterized by a contraction phase corresponding to the duration of the stimulation (500ms) followed by a relaxation phase after the end of the stimulation. The same phenomenon with similar temporal features was detectable also in single dissociated muscle fibers where a change in shape, again reminiscent of a slow contraction, occurred in the fiber region nearest to the electrode tip while leaving the rest of the fiber unmodified (Figure 4).

As an additional consequence of the electrical field, non-adherent cells that populate the culture dishes were induced to move and showed a slow continuous movement during stimulation approaching the electrode tip (Figure 5).

Fluorescence Microscopy Reveals Mechanisms Underlying the Cell Change in Shape

The physiological changes induced by the stimulation in muscle fibers were studied by means of fluorescent probes. The parameters analyzed were transmembrane potential monitored by means of Di-8-ANEPPS staining of the fibers, and cytosolic calcium ion concentration monitored with a Fluo4 labeling. For both parameters, a comparison was carried out with the changes induced by classical supra-threshold bipolar stimulation with two electrodes able to induce action potential and trigger contraction.

1. Membrane potential changes during stimulation

Classical single pulse field stimulation induced a change in membrane potential which was very fast, close to the limits of time resolution of the detection system, based on fluorescent light emission of Di-8-ANEPPS and video camera recording. Such fast changes are shown in Figure 6A and likely correspond to action potentials as also confirmed by the accompanying transient change in calcium concentration (see below).

The high frequency and low power stimulation determined slow membrane potential change which lasted for the whole duration of the stimulation phase and was followed by a slow reversal in the interval after the end of the stimulation (Figure 6B). The amplitude of the total change recorded for the membrane potential was about 8% in the membrane region closest to the electrode tip and decreased progressively along the fiber as illustrated in the Figure 7. Thus, with the high frequency stimulation, the cell membrane appeared to go through a charging process toward a steady state level during the stimulation phase, after that the potential slowly returned to its initial level.

2. Cytosolic calcium concentration changes during stimulation

Classical single pulse field stimulation, leading to a fast variation of the membrane potential as described above, triggered the voltage dependent process of calcium release from the sarcoplasmic reticulum and induced a rise in cytosolic calcium concentration. The time course of this process was totally independent of the stimulation timing and lasted about one hundred milliseconds, with a fast rising phase (<20ms) and a slower (<100ms) decay phase as shown in Figure 8A. From a spatial point of view, the process involved the whole muscle fiber, from one end to the other one, so allowing the activation of contraction along the fiber.

High frequency stimulation showed the peculiar property to focus its effects on a limited region of the fiber, inducing increase of calcium concentration mainly in the segments facing the stimulation electrode with a progressively decaying effect along the fiber as shown in Figure 9.

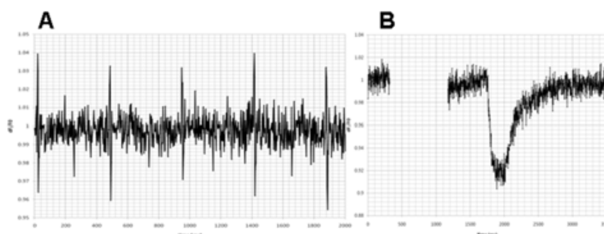

Fig. 6 *Change in membrane potential as monitored by fluorescence of Di-8-ANEPPS. A) Fast changes related to single pulse stimulation, likely corresponding to action potential. B) Long lasting change due to application of high frequency low power electrical field. On the abscissa a small division corresponds to 40 ms in A and 100 ms in B*

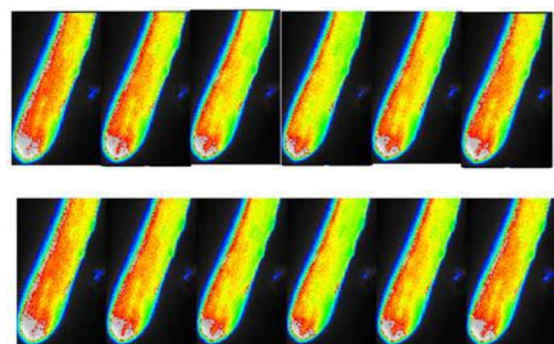

Fig. 7 *Spatial distribution of the changes in fluorescent light emission of a muscle fiber loaded with Di-8-ANEPPS to optically monitor membrane potential before, during and after AC stimulation. The blue spot indicates the position of the electrode. Changes in potential are proportional to emitted green light.*

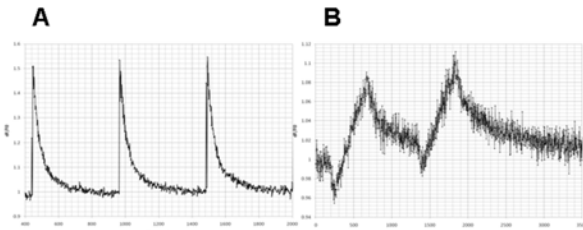

Fig. 8 *Changes in fluorescent light emission of a muscle fiber loaded with Fluo4 to optically monitor cytosolic free calcium concentration. A) Changes determined by single pulse bipolar stimulation, likely corresponding to transients following action potentials. B) Slow and low amplitude changes induced by high frequency low power electrical field application. A small division on the abscissa corresponds to 40 ms on A and 100 ms on B*

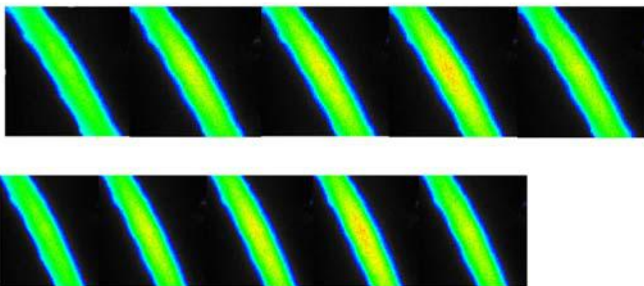

Fig. 9 *Spatial distribution of the changes in fluorescent light emission of fibers loaded with Fluo4 to optically monitor cytosolic free calcium concentration. A region of the fiber close to the electrode (not visible) is shown in sequential images. Calcium concentration is proportional to the emitted red light.*

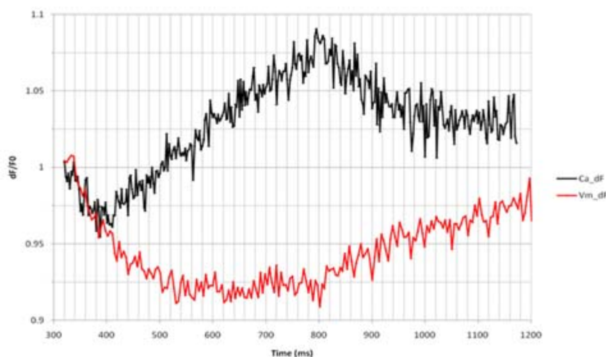

Fig. 10 *Time course of the fluorescent signals corresponding to membrane potential (red) and cytosolic calcium concentration (black) during the application of the high frequency low power electrical field for 500 ms, starting at 350 ms. Note that the electrical field induces a slow depolarization which is accompanied by a slow and continuous increase of calcium concentration.*

The time course of the increase of calcium concentration was also completely different from that produced by bipolar stimulation as, after the onset of the stimulation, a fast initial deflection in the fluorescence signal was followed by a continuous almost linear rising phase in the fluorescence signal lasting for the duration of the stimulation. A recovery phase with a decrease in the fluorescent signal was detected after the stimulation end, as shown in Figure 8B. The amplitude of the fluorescent signal was also very different as it reached a 50% increase for the bipolar stimulation and only a 10% with the high frequency stimulation. The temporal relation between the changes in membrane voltage gradient and the changes in cytosolic calcium concentration is shown in Figure 10. Importantly, the time course and the amplitude of the cytosolic calcium increase was similar in the presence or in the absence of calcium in extracellular medium, thus indicating that calcium was released from intracellular stores (data not shown).

Discussion

The results obtained in the present study provide the first description of the behavior of muscle fibers exposed to a high frequency (4-64 MHz) oscillating electrical field. The choice of this specific frequency range is related with the recent development of a commercial stimulator (Rexonage™, Telea), which is applied in many surgical and medical tasks and utilizes a well defined and patented frequency range. As outlined in the Introduction many studies have investigated the responses of cells and tissues to oscillating electrical fields. The direct effects of oscillating electrical fields are attributed to dielectric dispersion, i.e. to the variations of the permittivity of a dielectric material in relation to the frequency of the applied electric field.

In this study, an excitable tissue such as skeletal muscle was exposed to an oscillating electrical field and its behavior which depends partly on phenomena of β-dispersion (Maxwell Wagner effect, see [1, 8]) was described in detail. Experiments were carried out using a thin needle electrode to apply the electrical field with high spatial resolution and two distinct ranges of voltage. Whereas at high voltage the main effect of the field application was a fast and sharp cut of the muscle fiber or of the fiber bundle in a position adjacent to electrode tip, at low voltage the field application induced slow changes in muscle fiber shape. Such changes in shape were reminiscent of a contraction, but they developed in a continuous way for the whole period of application of the electrical field, did not involve the whole fiber and remained localized in a region close to the electrode tip. Such changes in shape might be compared to the deformation previously observed in red blood cells exposed to an oscillating electrical field in the MHz range [5, 6].

Muscle fibers, however, are able to modify actively their shape and size, when a contraction is triggered, making important to separate the direct effects of electrical field from active contractile response. Fluorescent probes were employed to follow the changes in membrane potential and in cytoplasmic free calcium concentration and this allowed us to demonstrate that the response to a low voltage electrical field was not a contraction. Actually, the membrane was slowly and progressively depolarized for the whole duration of the field application without reaching a threshold sufficient to trigger an action potential. Apparently, the changes in membrane potential were not sufficient to trigger the opening of the voltage gated sodium channels. Such slow and subliminar depolarization was followed by a small but progressive increase in free calcium concentration.

Experiments carried out in the absence of calcium in the extracellular medium showed that the origin of such calcium increase was likely localized in intracellular calcium stores. We could thus hypothesize that the application of the high frequency electrical field induced a membrane depolarization not sufficient to cause the opening of voltage gated sodium channels, but sufficient to induce the opening of ryanodine receptors calcium channels at the terminal cisternae of the sarcoplasmic reticulum and to cause the release of small amount of calcium. Such increase of intracellular calcium concentration represents a major difference if the effects on muscle fibers are compared to the effects observed in not excitable cells, in particular red blood cells red blood cells [5, 6].

The results obtained are rich of potential applications. At high power oscillating electrical fields are able to cut the membrane and the whole cell in a very fast and effective way. The damage is extremely limited, not only adjacent cells, but even the adjacent region of the same muscle fibers remain not affected. At low power, the oscillating electrical fields are able to cause a prolonged calcium accumulation in the cytosol at a level sufficient to elicit a small contractile response and avoid triggering of a full contraction. A long lasting and low increase of calcium concentration in the cytoplasm is likely an optimal signal to activate calcium-dependent intracellular signaling pathways. For example calmodulin-dependent kinase and phosphatase are known to be activated by low and continuous increase of calcium concentration better than by fast and large transients [3, 9]. In this view, the present study opens the way to further work aimed to assess whether such activation of signaling pathways really occur and to understand completely the possible application of high frequency electrical fields to stimulate muscle fiber plasticity.

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