Amplitude modulation of nuclear Ca\(^{2+}\) signals in human skeletal myotubes: A possible role for nuclear Ca\(^{2+}\) buffering

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Abstract

Video-rate confocal microscopy of Indo-1-loaded human skeletal myotubes was used to assess the relationship between the changes in sarcoplasmic ([Ca\(^{2+}\)]\(_{S}\)) and nuclear ([Ca\(^{2+}\)]\(_{N}\)) Ca\(^{2+}\) concentration during low- and high-frequency electrostimulation. A single stimulus of 10 ms duration transiently increased [Ca\(^{2+}\)] in both compartments with the same time of onset. Rate and amplitude of the [Ca\(^{2+}\)] rise were significantly lower in the nucleus (4.0- and 2.5-fold, respectively). Similarly, [Ca\(^{2+}\)]\(_{N}\) decayed more slowly than [Ca\(^{2+}\)]\(_{S}\) (mono-exponential time constants of 6.1 and 2.5 s, respectively). After return of [Ca\(^{2+}\)] to the prestimulatory level, a train of 10 stimuli was applied at a frequency of 1 Hz. The amplitude of the first [Ca\(^{2+}\)] transient was 25% lower than that of the preceding single transient. Thereafter, [Ca\(^{2+}\)]\(_{S}\) increased stepwise to a maximum that equalled that of the single transient. In contrast to [Ca\(^{2+}\)]\(_{S}\), [Ca\(^{2+}\)]\(_{N}\) then increased to a maximum that was 2.3-fold higher than that of the single transient and equalled that of [Ca\(^{2+}\)]\(_{S}\). In the nucleus, and to a lesser extent in the sarcoplasm, [Ca\(^{2+}\)] decreased faster at the end of the stimulus train than after the preceding single stimulus (time constants of 3.3 and 2.1 s, respectively). To gain insight into the molecular principles underlying the shaping of the nuclear Ca\(^{2+}\) signal, a 3-D mathematical model was constructed. Intriguingly, quantitative modelling required the inclusion of a saturable nuclear Ca\(^{2+}\) buffer. Alterations in the concentration of this putative buffer had dramatic effects on the kinetics of the nuclear Ca\(^{2+}\) signal. This finding unveils a possible mechanism by which the skeletal muscle can adapt to changes in physiological demand.

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1. Introduction

Many cells display complex patterns of intracellular Ca\(^{2+}\) signalling in response to an extracellular stimulus [1,2]. Most importantly, the spatial and temporal organization of these intracellular Ca\(^{2+}\) signals differs greatly between stimuli and cell types. Increasing evidence shows that these differences are accomplished by differential expression of the various components of the Ca\(^{2+}\)-signalling machinery [3]. This flexibility makes the intracellular Ca\(^{2+}\) signal particularly suitable for the regulatory control of a diverse array of cellular activities including fertilization, proliferation, development, contraction, secretion, learning and memory.

Intracellular Ca\(^{2+}\) signals originate at the plasma membrane and/or the endoplasmic reticulum by the opening of specific types of Ca\(^{2+}\) channels and subsequently spread throughout the cytoplasm [3]. Apart from altering the activi-
ties of cytoplasmic Ca\textsuperscript{2+} effector molecules, these cytoplasmic Ca\textsuperscript{2+} signals are also efficiently transmitted into the mitochondria and nucleus, where they control the rates of ATP production [4] and gene transcription [5], respectively. Studies in recent years have identified Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) as the underlying principle in the rapid propagation of cytoplasmic Ca\textsuperscript{2+} waves [2]. As far as mitochondrial Ca\textsuperscript{2+} uptake is concerned, evidence is now provided that this occurs through a highly selective Ca\textsuperscript{2+} channel present in the mitochondrial inner membrane [6]. On the other hand, there is an ongoing debate regarding the mechanism by which the cytoplasmic Ca\textsuperscript{2+} signal is transmitted into the nucleus.

Some evidence favours the idea that Ca\textsuperscript{2+} diffuses into the nucleoplasm through nuclear pores [7]. Other evidence, however, suggests that Ca\textsuperscript{2+} is released into the nucleoplasm through Ca\textsuperscript{2+} channels that are present in the inner nuclear membrane and opened by the action of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) [8]. In this respect, it is intriguing that in many cell types the nuclear envelope forms long, double-membraned extensions deep into the nucleus [9]. More recently, these extensions have been implicated in the IP\textsubscript{3}-mediated release of Ca\textsuperscript{2+} in discrete areas of the nucleus [10]. Importantly, evidence has been provided that the IP\textsubscript{3}-operated Ca\textsuperscript{2+} channels of these intranuclear extensions, or nucleoplasmic reticulum, are more sensitive to IP\textsubscript{3} than those of the endoplasmic reticulum, thus allowing independent regulation of nuclear and cytoplasmic Ca\textsuperscript{2+} [11]. Finally, recent data indicate that mitochondria readily move in and out of the lumen of the intranuclear extensions [12]. Because energized mitochondria rapidly accumulate large amounts of Ca\textsuperscript{2+}, disease-related disturbances of the energy state of these organelles will significantly alter the shape of the nuclear Ca\textsuperscript{2+} signal and thus affect transcription regulation.

Thus far, there is no evidence for the presence of intranuclear extensions in the nuclei of skeletal muscle fibres. On the other hand, IP\textsubscript{3} receptors were demonstrated to be preferentially present in the nuclear fraction of cultured rat myotubes [13]. Elevations in extracellular K\textsuperscript{+} concentrations were shown to cause a biphasic increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}). Detailed analysis revealed that the first increase was fast and associated with contraction, whereas the second increase, which was most prominent in the nucleus, was slow, mediated by IP\textsubscript{3} and responsible for the activation of the transcription factor CREB (cAMP response element-binding protein; [13–15]). Similarly, a Ca\textsuperscript{2+} ionophore, known to cause a sustained increase in intracellular [Ca\textsuperscript{2+}]\textsubscript{i}, was found to increase the activity of the transcription factor NFAT (nuclear factor of activated T cells), in a primary culture of rabbit skeletal muscle [16,17]. The effect of the Ca\textsuperscript{2+} ionophore was mimicked by electrostimulation for 24 h [18].

In the latter study, measurement of the intracellular Ca\textsuperscript{2+} concentration revealed that this concentration invariably returned to the prestimulatory level in between the electrical stimuli. It should be noted, however, that the authors measured the average intracellular [Ca\textsuperscript{2+}] of a single myotube. Therefore, sustained increases in nuclear Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{n}), slightly higher than the prestimulatory level, might have been missed. In the present study we used videorate UV confocal imaging and electrostimulation of cultured human skeletal myotubes to assess the relationship between the changes in [Ca\textsuperscript{2+}]\textsubscript{n} (saroplasmic Ca\textsuperscript{2+} concentration) and [Ca\textsuperscript{2+}]\textsubscript{i} during low- and high-frequency electrostimulation. The data presented showed that [Ca\textsuperscript{2+}]\textsubscript{n} increased to significantly higher values upon changing from low- to high-frequency stimulation and quantitative modelling suggested an important role for nuclear Ca\textsuperscript{2+} buffering herein.

2. Materials and methods

2.1. Cell culture

Quadriceps needle muscle biopsies (25–50 mg) were obtained from healthy volunteers, as approved by the Dutch Committee on Medical Ethics. Myotube cultures were obtained as described previously [19]. In short, muscle samples were dissociated and incubated four times during 15 min in 20 ml Dulbecco’s phosphate-buffered saline containing 30 mg trypsin, 15 mg collagenase and 15 mg albumin. Satellite cells were spun down at 50 × g, resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Breda, The Netherlands) supplemented with 20% (v/v) foetal calf serum (FCS; Integro, Zaandam, The Netherlands) and allowed to proliferate for 4–5 days. Aliquots of the primary culture were stored in liquid nitrogen. For each measurement, an aliquot was plated on glass cover slips and cultured to confluence in DMEM with 20% (v/v) FCS. One day after reaching confluence, the medium was replaced by DMEM supplemented with 0.4% (v/v) Uitroser G (Invitrogen) to promote myoblast fusion into myotubes.

2.2. Confocal Ca\textsuperscript{2+} measurements

Three to five days after induction of myoblast fusion, cover slips were washed with incubation medium (pH 7.4) containing 125 mM NaCl, 10 mM NaHCO\textsubscript{3}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 5 mM KCl, 2 mM MgSO\textsubscript{4}, 1.8 mM CaCl\textsubscript{2}, 10 mM HEPES and 10 mM glucose and cells were loaded with the ratiometric fluorescent Ca\textsuperscript{2+} indicator, Indo-1, in the presence of 10 μM Indo-1/AM (Molecular Probes) for 30 min at 37°C. After loading, the cells were washed twice and incubated for an additional 15 min to remove excess dye and complete de-esterification. For acquisition of saroplasmic Ca\textsuperscript{2+} signals, cover slips were transferred to an incubation chamber (volume of 700 μl) mounted on the stage of an inverted microscope (Nikon Diaphot) attached to an OZ confocal unit (NORAN Instruments, Naarden, The Netherlands) with a high-power argon ion laser (Coherent Enterprise, Santa Clara, CA, USA). Indo-1 was excited at 351 nm and emission light was
collected using a Nikon ×40 water immersion objective (NA 1.2) with high UV transmission. Emission light was separated by a 455 DCLP dichroic mirror (Chroma Technology Corp, Brattleboro, VT, USA), and quantified at 405 ± 45 nm and 485 ± 45 nm using two photomultipliers (Hamamatsu Photonics Kabushiki Gasai, Japan). The confocal slit was chosen to give an axial resolution of 10 μm [20]. With this setting a workable signal-to-noise ratio at minimal laser intensity was achieved (output of 15 mW; equivalent to 28 μW at the back of the objective lens; [21]). Basal ratio signals were stable and no UV-induced deterioration of cells was observed during the measurements. The OZ hardware was controlled by the Image Acquisition software (version 1.5, Noran Instruments) running under IRIX 6.5 on an Indy workstation (Silicon Graphics Inc., Mountain View, CA, USA) equipped with 256 Mb of memory. Before starting the measurement, regions of interest (ROIs) were indicated in sarcoplasm and nucleus of the myotube. For background correction, an additional ROI was situated outside the myotube. During the measurement, the average fluorescence emission intensities from each ROI were calculated and individually stored in ASCII format for off-line background correction and ratioing.

3.1. [Ca2+] dynamics in sarcoplasm and nucleus following a single stimulus

In contrast to mouse myotubes and C2C12 cells [22,23], human myotubes did not display spontaneous [Ca2+] rises [21]. In previous work, we used elevations in extracellular [K+] to increase [Ca2+] in human myotubes [24]. However, this method does not allow high-frequency stimulation. Therefore, electrical field stimulation was applied in a more recent study [21]. It was found that electrically induced [Ca2+] rises occurred only in myotubes and not in myoblasts.

Here, we combined video-rate UV confocal imaging and electrostimulation to assess the relationship between the changes in [Ca2+]n and [Ca2+]s during low and high-frequency stimulation of human myotubes. Three to five days after induction of myoblast fusion, cells were loaded with the ratiometric UV Ca2+-indicator Indo-1. Myotubes were readily recognized by virtue of their elongated shape and the presence of multiple nuclei (Fig. 1A; left panel). Importantly, ratio images of unstimulated myotubes revealed no spatial differences in ratio signal (Fig. 1A; right panel and trace to the right). Fig. 1B shows that a single electrical stimulus (10 ms, 16 V) readily increased [Ca2+] in two ROIs, a sarcoplasmic one and a nuclear one, located in close proximity to each other (Fig. 1A; left panel). Within the time resolution of the measurement (33 ms), the onset of the [Ca2+]n rise was the same in both compartments. However, the maximal rate of [Ca2+] rise was significantly lower in the nucleus (rising speeds of 3.1 and 11.3 ratio units/s for [Ca2+]n and [Ca2+]s, respectively). In both compartments, the peak [Ca2+]n was reached within 100 ms, after which [Ca2+]n gradually declined to the prestimulatory level (Fig. 1C; left panel). The peak [Ca2+] n or amplitude, was significantly lower in the nucleus (0.29 ± 0.02 and 0.70 ± 0.05 ratio units for [Ca2+]n and [Ca2+]s, respectively) (Fig. 1D; column marked ‘single’). In both compartments, the decay phase of the [Ca2+] transient was adequately described by a mono-exponential function (R(t) = Rmax [1 − e −t/τ] + 1; R2 > 0.90), indicating the involvement of a single Ca2+ removal process [21,25,26]. The time constant (τ) for the decay phase was significantly higher for the nucleus (τ values of 6.1±0.30 s and 2.5±0.15 s for [Ca2+]n and [Ca2+]s, respectively), indicating that Ca2+ was less rapidly removed from this organelle (Fig. 1E; column marked ‘single’).
3.2. Frequency dependence of sarcoplasmic and nuclear \([Ca^{2+}]\) changes

To determine the dependence of the kinetics of the sarcoplasmic and nuclear \([Ca^{2+}]\) rises on stimulus frequency, we used a protocol consisting of a single stimulus followed by two trains of 10 stimuli, a first one at 1 Hz and a second one at 2 Hz (Fig. 1C). In between, \([Ca^{2+}]\) was allowed to return to the prestimulatory level (dashed line). The peak value of the first \([Ca^{2+}]\) rise during the 1 Hz stimulus train was 25% lower than that of the preceding single \([Ca^{2+}]\) transient (Fig. 1C, arrows with open heads in left and middle
During 1 and 2 Hz stimulation, [Ca\textsuperscript{2+}]\textsubscript{S} did not return to the prestimulatory level in between the stimuli and the peak value increased stepwise to a maximum, reached after three consecutive stimuli, that equalled that of the preceding single transient (0.68 ± 0.07 and 0.70 ± 0.05 ratio units, respectively) (Fig. 1D). Similarly, the peak value of the first [Ca\textsuperscript{2+}]\textsubscript{N} transient was 20% lower than that of the preceding single [Ca\textsuperscript{2+}]\textsubscript{N} transient (Fig. 1C; arrows with filled heads). In this compartment, however, the peak value increased stepwise to a maximum, reached after seven to eight consecutive stimuli, that was 2.3-fold higher than that of the preceding single [Ca\textsuperscript{2+}]\textsubscript{N} transient (0.68 ± 0.02 and 0.29 ± 0.02 ratio units, respectively) and equalled that of [Ca\textsuperscript{2+}]\textsubscript{S}. In the nucleus, [Ca\textsuperscript{2+}]\textsubscript{N} decreased faster at the end of the stimulus train than during the preceding single transient (time constants of 3.30 ± 0.34 s and 6.11 ± 0.30 s, respectively) (Fig. 1E).

The same was observed for the sarcoplasm (time constants of 2.07 ± 0.19 s and 2.50 ± 0.15 s for the [Ca\textsuperscript{2+}]\textsubscript{S} decay at the end of the first stimulus train and after the preceding single stimulus, respectively). The reduction in time constant for the decrease in [Ca\textsuperscript{2+}]\textsubscript{S} and [Ca\textsuperscript{2+}]\textsubscript{N} amounted to 17 and 46%, respectively.

After return of [Ca\textsuperscript{2+}]\textsubscript{S} to the prestimulatory level, a 2 Hz stimulus train was applied. Now, the peak value of the first [Ca\textsuperscript{2+}]\textsubscript{S} rise was 40% lower than that of the initial single [Ca\textsuperscript{2+}]\textsubscript{S} transient (Fig. 1C; arrows with open heads in left and right panel). However, upon repeated stimulation this value increased stepwise to a maximum, reached after five consecutive stimuli, that was not statistically different from that of the initial single transient (0.65 ± 0.06 and 0.70 ± 0.05 ratio units, respectively) (Fig. 1D). Similarly, the peak value of the first [Ca\textsuperscript{2+}]\textsubscript{S} rise was 41% lower than that of the initial single [Ca\textsuperscript{2+}]\textsubscript{N} transient (Fig. 1C; arrows with filled heads in left and right panel). Again, the peak value increased stepwise to a maximum, reached after 9–10 consecutive stimuli, that was markedly (2.0-fold) higher than that of the initial single transient (0.58 ± 0.07 and 0.29 ± 0.02 ratio units, respectively) and virtually equalled that of [Ca\textsuperscript{2+}]\textsubscript{S}. In both compartments, the rate of [Ca\textsuperscript{2+}]\textsubscript{S} decay was faster at the end of the 2 Hz stimulus train than at the end of the preceding 1 Hz stimulus train (time constants of 1.65 ± 0.05 s and 2.07 ± 0.19 s for the sarcoplasm and 2.40 ± 0.13 s and 3.30 ± 0.34 s for the nucleus, respectively) (Fig. 1E). Compared to the initial single stimulus, the reduction in time constant amounted to 34 and 61% for the decrease in [Ca\textsuperscript{2+}]\textsubscript{S} and [Ca\textsuperscript{2+}]\textsubscript{N}, respectively. For both sarcoplasm and nucleus the Indo-1 emissions at 405 and 485 nm were linearly correlated (p < 0.001). The fitted lines were virtually parallel, indicating that Indo-1 behaved the same in both cellular compartments (data not shown).

3.3. Three-dimensional model for amplitude modulation of nuclear Ca\textsuperscript{2+} signals

To gain insight into the molecular principles underlying the shaping of the nuclear Ca\textsuperscript{2+} signal, we developed a 3-D mathematical model using a minimal number of components required to quantitatively reproduce the experimental findings. The numerical data presented in Fig. 1D and E were obtained from equally sized ROIs located in close proximity to each other in the nuclear and sarcoplasmic compartment (Fig. 1A, left panel). The nuclear ROI consisted of a square, whose corners touched the boundary of the organelle (Fig. 2A). The lateral dimensions of this square were 10 μm × 10 μm. A square with identical dimensions was drawn in the sarcoplasm. Because the confocal slit was chosen to give an axial section thickness of 10 μm [20], this means that fluorescence light was sampled from two cubical volumes of interest (VOIs) with a side of 10 μm (Fig. 2B).

Geometrically, the nucleus was considered as a 3-D sphere, consisting of five concentric shells surrounded by five shells of sarcoplasm (Fig. 2C; grey area: projection on x-y plane shown). Shells were 1.5 μm apart and used to calculate [Ca\textsuperscript{2+}]\textsubscript{S}(x, y, z, t) = [Ca\textsuperscript{2+}]\textsubscript{S}(t) [Ca\textsuperscript{2+}]\textsubscript{S}(x, y, z, t) (the experimental geometry was accounted for by calculating [Ca\textsuperscript{2+}]\textsubscript{S}(x, y, z, t) at time t, from the full 3-D multi-shell model followed by integration across the corresponding VOI (using appropriate boundary conditions). Earlier studies demonstrated that during each electrical stimulus a constant fraction α of the Ca\textsuperscript{2+} in the SR is released and that this fraction decreases with increasing stimulus intensity [27,28]. This is reflected in our model by using α = 0.5 for a single stimulus, α = 0.4 for a 1 Hz stimulus and α = 0.25 for a 2 Hz stimulus.

Previous work in rat soleus muscle revealed a perinuclear localization of the sarcoplasmic reticulum, suggesting a high...
abundance of SERCA pumps in this region [29–31]. Immuno-
cytocchemical analysis confirmed that this was also the case for the human myotubes used in this study (Fig. 2D, arrow). Therefore, the Ca\(^{2+}\) removal rate was considered to be highest around the nucleus and to decrease linearly with the distance from the nuclear membrane. This was accounted for in the model by a decrease in \(\lambda\), the first-order time constant that governs the process of Ca\(^{2+}\) removal, from 1.2 to 0.24 s when going from shell 10 to shell 6 (Fig. 2F, filled circles). Under all conditions, the rate of Ca\(^{2+}\) removal in the sarcoplasm exceeded that in the nucleus. This suggests that the rate of Ca\(^{2+}\) removal from the nucleus is determined by that in the sarcoplasm. Therefore, our model does not include active Ca\(^{2+}\) removal in the nucleus. Consequently, \(\lambda\) was set to zero in the nucleus (shells 1–5). It was further assumed that Ca\(^{2+}\) diffusion in the nucleus was somewhat faster than in the sarcoplasm (\(D_N = 5.0 \mu m^2/s\), \(D_S = 3.0 \mu m^2/s\)).

Because strategically localized mitochondria have dra-
matic effects on the propagation of intracellular Ca\(^{2+}\) sig-
als [32,33], we determined their subcellular localization by expressing a mitochondria-targeted variant of yellow fluores-
cent protein (COX8-EYFP) using a transfection method described previously [34]. Fig. 2E (asterisks indicate nucleus) shows the absence of a near perinuclear clustering of these organelles in human skeletal myotubes. Similar results were obtained with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), a cationic dye that accumulates in mitochondria (not shown). These find-
ings make it unlikely that mitochondria form an effective barrier for the sarcoplasmic Ca\(^{2+}\) signal to propagate into the nucleus.

In agreement with earlier findings, the nuclear membrane was considered to be semi-permeable for Ca\(^{2+}\) [31,35–37]. Mathematically, this was achieved by using a reduced Ca\(^{2+}\)
diffusion constant \(D_{SN}\) of 1.0 \(\mu\text{m}^2/\text{s}\) between the sarcoplasm and nucleus (i.e. between shells 5 and 6; Fig. 2F, open circles). Considering the above assumptions, the \(Ca^{2+}\) flux in the nucleus \(J_N\) is entirely driven by diffusion:

\[
J_N(x, y, z, t) = -D_{SN} \nabla C(x, y, z, t)
\]  

(1)

On its turn, the \(Ca^{2+}\) flux in the sarcoplasm \(J_S\) is determined by \(Ca^{2+}\) release, diffusion and extrusion:

\[
J_S(x, y, z, t) = \text{release} - D_{SN} \nabla C(x, y, z, t) - \text{extrusion}
\]  

(2)

The continuity equation, stating that the total amount of \(Ca^{2+}\) is preserved, gives:

\[
\nabla \cdot J = \frac{\partial (\nabla \cdot C)}{\partial t} + \frac{\partial C}{\partial t}
\]  

(3)

Substitution of \(J\) in Eqs. (1) and (2) in Eq. (4) gives the diffusion equation:

\[
\frac{\partial C(x, y, z, t)}{\partial t} = D \left( \frac{\partial^2 C(x, y, z, t)}{\partial x^2} + \frac{\partial^2 C(x, y, z, t)}{\partial y^2} + \frac{\partial^2 C(x, y, z, t)}{\partial z^2} \right)
\]  

(4)

Due to symmetry this equation can be expressed in spherical coordinates:

\[
\frac{\partial C(r, t)}{\partial t} = D \left( \frac{\partial^2 C(r, t)}{\partial r^2} + \frac{2 \partial C(r, t)}{r \partial r} \right)
\]  

(5)

To simulate an electrical stimulus, \([Ca^{2+}]_S\) was increased within a single time step (1 ms) to 1 \(\mu\text{M}\), a concentration typical for skeletal muscle [38]. The set of Eqs. (1)-(5) was solved using Newton-integration.

3.4. Quantitative modelling requires nuclear \(Ca^{2+}\) buffering

During experiments the ratio between the sarcoplasmic and nuclear peak \([Ca^{2+}]\) \((R_{max,S}/R_{max,N})\) declined from
2.41 (100%) for the single transient, to 1.03 (43%) for the 1 Hz stimulus train and 1.12 (46%) for the 2 Hz stimulus train, whereas in the model (BUFFER, DSN = 1.0) these ratios were 1.02 (100%), 1.02 (100%) and 1.03 (101%), respectively. These findings demonstrate that (i) the model was able to qualitatively predict the amplitude reduction in [Ca2+]N, as a function of electrostimulation frequency, but (ii) failed to reproduce the differences in µN and µS observed during experiments.

The quantitative performance of the model was considerable improved by introducing an immobile Ca2+ buffer B in the nuclear shells at a total concentration ([B]T ) of 1.5 µM (Fig. 2F, open squares). The buffer was assumed to display first order reaction kinetics, which was accounted for by adding d[B]Ca2+[B]/dt = Koff[B][Ca2+]N - Koff[B]Ca2+[B] to the right hand side of Eq. (1), with Koff = 4 µM/s, Koff = 0.5 s-1 and Koff = 0.125 µM. Models including this nuclear buffer (+BUFFER), predicted the stimulus-dependent decrease in Rmax/[Ca2+]N and µN/µS much better than those without nuclear buffer (+BUFFER), irrespective of the value of DSN (Fig. 3A and B and Table 1).

The best fit between experiment and model was obtained using [B]T = 1.5 µM and DSN = 1 µm2/s (Fig. 3C). The latter value reflects a three-fold reduction in Ca2+ diffusion (from 4.21 (100%) for the 1 Hz stimulus train and 1.40 (58%) for the 2 Hz stimulus train, whereas in the model (BUFFER, DSN = 1.0) these ratios were 1.02 (100%), 1.02 (100%) and 1.03 (101%), respectively. These findings demonstrate that (i) the model was able to qualitatively predict the amplitude reduction in [Ca2+]N, as a function of electrostimulation frequency, but (ii) failed to reproduce the differences in µN and µS observed during experiments.

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4. Discussion

Video-rate confocal microscopy of fura-2-loaded human skeletal myotubes was used to gain insight into the temporal and quantitative relationship between the sarcoplasmic and nuclear Ca2+ concentration changes following electrical stimulation. Each stimulus evoked a transient increase in [Ca2+]S and [Ca2+]N and it was observed that [Ca2+]S, but not [Ca2+]N, reached a considerably higher value during repetitive [Ca2+]S transients as compared to a single [Ca2+]S transient. Using quantitative mathematical modelling we investigated the involvement of semi-permeability of the nuclear membrane, inhomogeneous sarcoplasmic Ca2+ pumping and high-affinity nuclear Ca2+ buffering in this amplitude modulation (AM).

4.1. Validation of the use of Indo-1 to monitor electrically evoked changes in [Ca2+]S

It was observed that Indo-1 emission signals (especially at 405 nm) were higher in the nucleus than in the sarcoplasm. This property allowed precise identification of sarcoplasmic and nuclear ROIs. Importantly, unstimulated myotubes displayed no spatial differences in Indo-1 ratio. This excludes the presence of a standing gradient between [Ca2+]S and [Ca2+]N and makes it unlikely that our results are biased by dye compartmentalization. Others have found that steady-state Indo-1 ratios, in contrast to Fluo-3 signals, were insensitive to pH, temperature and dye concentration and did not differ between cytosol and nucleoplasm for Ca2+ concentrations ranging from 10 to 10000 nM [39]. Therefore, we conclude that the observed kinetic differences between [Ca2+]S and [Ca2+]N are unlikely to reflect Indo-1 associated artefacts.

4.2. A 3-D model for amplitude modulation of nuclear Ca2+ signals

When comparing the sarcoplasmic and nuclear Ca2+ changes evoked by a single electrical stimulus with those evoked by a subsequent train of stimuli at a frequency of 1 Hz, two major differences were observed. First, [Ca2+]N stepwise increased to a value that was 2.3-fold higher than following a single stimulus, whereas the maximum value of [Ca2+]S remained the same. To better understand the mechanism underlying this frequency-dependent AM of [Ca2+]N, a 3-D mathematical model was developed from which data were sampled in agreement with the experimental geometry. In our model, each electrical stimulus was simulated by an instantaneous (within 1 ms) increase of [Ca2+]S to 1 µM. This direct coupling reflects the highly efficient physiological interaction between the dihydropyridine receptor (DHPR) voltage sensor and the ryanodine-sensitive Ca2+ release channel (RyR) on the sarcoplasmic reticulum (SR [38]). Second, the amplitude of the first [Ca2+]N transient of the 1 Hz train was 25% lower than that of the preceding single [Ca2+]S transient, whereas that of the subsequent 2 Hz train was even...
Fig. 3. Modeling of sarcoplasmic and nuclear calcium changes during electrical field stimulation. (A) Ratio between the maximal amplitude of the nuclear and sarcoplasmic Ca\textsuperscript{2+} signal, calculated from the ratio between the maximal Indo-1 ratio in nucleus and sarcoplasm (\(R_{\text{max,S}}/R_{\text{max,N}}\)), for stimuli of increasing frequency. The open square represents experimental data whereas the other symbols indicate model simulations for different values of \(D_{\text{SN}}\) (\(\mu\text{m}^2/\text{s}\)), the Ca\textsuperscript{2+} diffusion coefficient at the boundary between sarcoplasm and nucleus. Experimental data was normalized to one and therefore the ratio was calculated from \((R_{\text{max,S}} - 1)/(R_{\text{max,N}} - 1)\). Ratios for the 1 and 2 Hz stimulus were expressed as percentage of the ratio for a single stimulus (data from Table 1). Inclusion of a nuclear Ca\textsuperscript{2+} buffer (+BUFFER) was required for quantitative simulation of the experimental results. (B) Similar to (A) but now for \(\mu\text{N}/\mu\text{S}\), the ratio between the mono-exponential time constant of Ca\textsuperscript{2+} in nucleus and sarcoplasm. Nuclear Ca\textsuperscript{2+} buffering was required for quantitative simulation. (C) Simulated experiment for a model with nuclear Ca\textsuperscript{2+} buffering (\([B] = 1.5\mu\text{M}\)) and a reduced Ca\textsuperscript{2+} diffusion across the nuclear membrane (\(D_{\text{SN}} = 1.0\mu\text{m}^2/\text{s}\)). Open and filled arrows indicate a reduction in amplitude of the first Ca\textsuperscript{2+} transient at higher stimulus frequency in the sarcoplasm and nucleus, respectively. The latter was also observed during experiments. Simulation parameters can be found in Table 1 and details are in the Section 3. (D) Magnification of the rising phase of the transient in the left panel of (C) for different values of \(D_{\text{SN}}\). Simulated traces were down sampled to 30 Hz to allow faithful comparison with experiments. (E) Time course of the concentration of free nuclear Ca\textsuperscript{2+} buffer [B] during a single, 1 and 2 Hz stimulus. For details see Section 3.
This indicates that the amplitude of an electrically evoked \([\text{Ca}^{2+}]_S\) transient depends on whether or not a myotube is stimulated before. This phenomenon can be explained in several ways. For instance, the filling state of the SR might be decreased as a consequence of prior stimulation. Another possibility is that the DHPR and/or RyR are desensitized as a consequence of prior stimulation. In this context, it has been found that inorganic phosphate exerts a negative feedback on the RyR [27,28]. To account for the observed decrease in amplitude when going from the initial single stimulus to the first \([\text{Ca}^{2+}]_S\) transient of the 1 Hz pulse train and subsequent 2 Hz pulse train, we included a term \(\alpha\) in our mathematical model. The lower the value of \(\alpha\), the less \(\text{Ca}^{2+}\) is released during each electrical stimulus. For an optimal fit of the experimental data, values of \(\alpha\) of 0.5, 0.4 and 0.25 were required for the initial single stimulus and the first (1 Hz) and second (2 Hz) pulse train, respectively. With these values of \(\alpha\), the model not only reproduced the progressive decrease in amplitude of the first \(\text{Ca}^{2+}\) transient of the two stimulus trains but also the maximal \([\text{Ca}^{2+}]_S\) reached during the successive stimulations. In other words, when \(\alpha\) was kept constant (for instance 0.5), the amplitude of the first \(\text{Ca}^{2+}\) transient of each of the two stimulus trains was the same as that of the initial single stimulus, whereas the maximal amplitude reached during each of the stimulus trains was significantly higher than that of the initial single stimulus.

Immunocytochemistry revealed a preferential perinuclear staining for SERCA pumps. Similar results were obtained with cardiomyocytes [40] and coronary smooth muscle cells [41]. Therefore in the model, sarcoplasmic \(\text{Ca}^{2+}\) removal (accounted for by a single time constant \(\lambda\)) was considered to be inhomogeneous, being highest just outside the nucleus. Quantitative simulations required a value of \(\lambda = 1.2\) s in close agreement with experimental findings. Under all conditions, \(\text{Ca}^{2+}\) removal was faster in the sarcoplasm as compared to the nucleus. This suggests that the rate of sarcoplasmic \(\text{Ca}^{2+}\) removal determines the \(\text{Ca}^{2+}\) removal rate in the nucleus. This finding supports the idea that \(\text{Ca}^{2+}\) removal from the nucleus does not involve the action of \(\text{Ca}^{2+}\)-ATPases and/or \(\text{Ca}^{2+}\)-exchangers on the inner membrane of the nuclear envelope but rather reflects the diffusion of \(\text{Ca}^{2+}\) from the nucleus via nuclear pore complexes (NPCs) into the sarcoplasm [35,36,42]. Based on these considerations, we modelled a first-order \(\text{Ca}^{2+}\) removal process only in the sarcoplasm and not in the nucleus.

Skeletal myotubes are densely packed with mitochondria arranged in rows along the length of the cell [43]. Mitochondria can accumulate significant amounts of \(\text{Ca}^{2+}\) thereby changing the spatio-temporal kinetics of the intracellular \(\text{Ca}^{2+}\) signal. The present finding that the maximal increase in \([\text{Ca}^{2+}]_N\) was not different between the pulse trains and the preceding single stimulus suggests that mitochondrial \(\text{Ca}^{2+}\) buffering was already maximal during the single stimulus and can therefore not be responsible for the lower increase in \([\text{Ca}^{2+}]_N\) during this stimulus as compared to the stimulus trains. Moreover, the onset of the increase in \([\text{Ca}^{2+}]_N\) did not differ from that in \([\text{Ca}^{2+}]_S\) within the sampling interval of our recordings (33 ms). In contrast to our study, a significant delay (~300 ms) was observed in carbachol-stimulated parotid acinar cells [33]. In these cells, the majority of mitochondria are localized around the nucleus and it was found that inhibitors of mitochondrial \(\text{Ca}^{2+}\) uptake abolished this delay suggesting the involvement of these organelles in propagation of the \(\text{Ca}^{2+}\) signal from the cytosol into the nucleus.

The absence of such a delay in the present study then indicates that mitochondria localized in the perinuclear region in skeletal myotubes do not accumulate sufficient amounts of \(\text{Ca}^{2+}\) to interfere with \(\text{Ca}^{2+}\) signal propagation from the sarcoplasm to the nucleus. We therefore did not include perinuclear mitochondrial \(\text{Ca}^{2+}\) uptake in our mathematical model. Our mathematical model considers the decay in \([\text{Ca}^{2+}]_S\) as a first order process characterized by a single rate-constant \(\lambda\), accounting for all removal processes in the sarcoplasm (including SERCA-mediated \(\text{Ca}^{2+}\) uptake and mitochondrial \(\text{Ca}^{2+}\) uptake). Because mitochondrial \(\text{Ca}^{2+}\) uptake stimulates mitochondrial ATP production [34] and thus fuelling of the sarcoplasmic \(\text{Ca}^{2+}\) pumps [21], we can model the effect of reduced mitochondrial \(\text{Ca}^{2+}\) uptake on the sarcoplasmic and nuclear \(\text{Ca}^{2+}\) changes by increasing \(\lambda\). The outcome can be tested using inhibitors of mitochondrial \(\text{Ca}^{2+}\) uptake.

Experimental data obtained with cardiac myocytes lead to the conclusion that \(\text{Ca}^{2+}\) diffused freely through NPCs at low cytosolic \(\text{Ca}^{2+}\) and that this diffusion was partially restricted at high cytosolic \(\text{Ca}^{2+}\) [40]. Our experiments show that the maximal amplitude of \([\text{Ca}^{2+}]_N\) remained the same when changing from a single stimulus to a train of stimuli. Therefore, our mathematical model required only a single value for the diffusion constant describing the diffusion across the nuclear envelope (\(D_N = 1 \mu^2/s\)). This value was three-fold lower than that for the \(\text{Ca}^{2+}\) diffusion constant in the sarcoplasm (\(D_S = 3 \mu^2/s\)) and five-fold lower than that for the \(\text{Ca}^{2+}\) diffusion constant in the nucleus (\(D_N = 5 \mu^2/s\)). Increasing the value of \(D_N\) from 0.5 to 2.8 \(\mu^2/s\) at a constant maximal amplitude of \([\text{Ca}^{2+}]_N\), increased both the rate of increase and the maximal amplitude of \([\text{Ca}^{2+}]_N\) (Fig. 3D). In contrast, this manoeuvre did not alter the time of onset of the \([\text{Ca}^{2+}]_N\) transient (Fig. 3D) nor improve the quantitative fit between model and experiment (Fig. 3A and B).

Previous work with hippocampal neurons revealed that electrical field stimulation led to a rapid translocation of calmodulin from the cytoplasm to the nucleus leading to a 2.4-fold increase within 42 s after the onset of stimulation [44]. However, quantitative modelling of the data obtained with skeletal myotubes required inclusion of a \(\text{Ca}^{2+}\) buffer (B) in the nuclei of unstimulated myotubes. This requirement mainly concerned the first single stimulus in that it allowed adjustment of the maximal amplitude of \([\text{Ca}^{2+}]_N\) and rate of nuclear \([\text{Ca}^{2+}]_N\) decay to experimental values. In contrast, inclusion of this buffer had virtually no effect on the kinetics of \([\text{Ca}^{2+}]_S\). During 1 Hz stimulation, however, the model predicts that the nuclear \(\text{Ca}^{2+}\) buffer is almost saturated. As a consequence, \([\text{Ca}^{2+}]_N\) increases to a value...
that is markedly higher than observed for a single stimulus. From the fact that our model fully reproduces the sarcoplasmic and nuclear Ca\textsuperscript{2+} changes it can be concluded that the nuclear buffer capacity does not significantly change during the electrical stimulations. In other words, our model does not require a mobile Ca\textsuperscript{2+} buffer. Therefore, our model predicts that calmodulin molecules that enter the nucleus during electrical stimulation do not significantly add to the nuclear buffer capacity. In the presence of a nuclear Ca\textsuperscript{2+} buffer, changing \(\Delta\psi\text{NS} \) had only minor effects. This indicates that the presence of the buffer, and not the semi-permeability of the nuclear membrane, is the dominant process in shaping \([\text{Ca}^{2+}]\text{N}\). Evidence for the presence of nuclear Ca\textsuperscript{2+} buffers was obtained in pancreatic acinar cells [45], rat hepatocytes [46] and dopaminergic neurons [47]. Currently, we can only speculate about the molecular identity of the putative nuclear Ca\textsuperscript{2+} buffer in human skeletal myotubes because its predicted properties \((K_d = 0.125 \, \text{mM}, [B]_T = 1.5 \, \text{mM})\) do not match with those of Indo-1 or one of the known nuclear Ca\textsuperscript{2+} binding proteins.

4.3. Physiological implications

Our experimental results indicate that although \([\text{Ca}^{2+}]\text{N}\) is directly derived from \([\text{Ca}^{2+}]\text{S}\), the frequency of the electrical stimulus determines whether AM of \([\text{Ca}^{2+}]\text{N}\) occurs. It is tempting to speculate that such decoupling between sarcoplasmic and nuclear Ca\textsuperscript{2+} signals allows low-frequency \([\text{Ca}^{2+}]\text{S}\) signals to stimulate contraction without transcription activation, whereas high-frequency \([\text{Ca}^{2+}]\text{S}\) signals activate both contraction and transcription. This mechanism might underlie the observation that the temporal pattern of electrical input (i.e. motoneuron firing) determines whether a muscle fibre becomes slow oxidative or fast glycolytic [48].

One can hypothesize that the cell can adapt the Ca\textsuperscript{2+} signalling mechanism to specific physiological requirements by varying the expression levels and/or kinetic properties of its constituents such as SERCA pumps, nuclear Ca\textsuperscript{2+} buffers and/or NPCs on a relatively fast (e.g. Ca\textsuperscript{2+}-mediated closure of NPCs) or slow timescale (e.g. SERCA isoform switching; [49]). This idea is supported by the observation in human skeletal muscle that SERCA activity and subtype expression depend on fibre type [50] and that transcription rates of SERCA and phospholamban genes change in response to chronic stimulation [51]. On the other hand, decoding of the Ca\textsuperscript{2+} signal might be altered by expression of CaMKII isoforms with different Ca\textsuperscript{2+}-dependent kinetics [52].

Although a nucleoplasmic reticulum has been demonstrated in a hepatic cell line [10], its existence in human skeletal muscle still awaits discovery. In this context, the present data and modelling results do not provide evidence for the presence of a nucleoplasmic reticulum in human skeletal myotubes. Our quantitative simulations predict that AM of \([\text{Ca}^{2+}]\text{N}\) requires both semi-permeability of the nuclear membrane and nuclear Ca\textsuperscript{2+} buffering. However, the Ca\textsuperscript{2+} permeability of the nuclear membrane appears to be of minor importance in human skeletal myotubes.


