Excitation-contraction coupling of cultured human skeletal muscle cells and the relation between basal cytosolic Ca\textsuperscript{2+} and excitability

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Summary Cultured human skeletal muscle cells are frequently used as a model to study muscle pathology, in which Ca\textsuperscript{2+}-homeostasis might be affected. However, their excitation-contraction (E–C) coupling has been poorly investigated.

In order to elucidate E–C coupling of cultured muscle cells, we activated the acetylcholine receptors, voltage-dependent Na\textsuperscript{+} channels, dihydropyridine receptors or ryanodine receptors both in the presence and absence of external Ca\textsuperscript{2+}, as well as after specific inhibition, and measured the effects on the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) using Fura-2. Furthermore, we examined the excitability of these cells during iterative high (125 mM) K\textsuperscript{+} stimulation with various repolarisation intervals.

The resting [Ca\textsuperscript{2+}], in muscle cells of controls is about 130 nM. Acetylcholine, veratridine, high K\textsuperscript{+} and caffeine elicit dose-dependent Ca\textsuperscript{2+} transients, which are independent of extracellular Ca\textsuperscript{2+} and can be inhibited by α-bungarotoxin, tetrodotoxin, nifedipine or ryanodine.

During repetitive K\textsuperscript{+} stimulation, the excitability of the muscle cells depends on the repolarisation interval between successive stimulations. Upon shortening the repolarisation time the Ca\textsuperscript{2+} transients become smaller and slower. Thereby, the basal [Ca\textsuperscript{2+}], rises, the Ca\textsuperscript{2+} response amplitude declines and both the half-increase and half-decay time increase. However, if the basal [Ca\textsuperscript{2+}] equals the resting [Ca\textsuperscript{2+}], the initial Ca\textsuperscript{2+} response can be recovered. The intracellular pH of 7.23, measured by BCECF, is unaffected by repeated K\textsuperscript{+} stimulation, whatever the repolarisation interval was.

In conclusion, cultured human skeletal muscle cells possess a ‘skeletal muscle type’ of E–C coupling and their excitability at iterative stimulation is set by their basal [Ca\textsuperscript{2+}],.

INTRODUCTION

The excitation-contraction (E–C) coupling mechanism of skeletal muscle in vivo is a cascade of events in which sequentially acetylcholine receptors (AChR) in the neuromuscular junction, sarcolemmal tetrodotoxin-sensitive voltage-operated Na\textsuperscript{+} channels (TTXR), T-tubular dihydropyridine receptors (DHPR) and ryanodine receptors (RyR),
i.e. Ca\textsuperscript{2+}-release channels, in the terminal cisternae of the sarcoplasmic reticulum (SR) are involved [1,2]. The DHPR may serve a dual function as slow-activated voltage-dependent Ca\textsuperscript{2+}-channel [3] and as voltage sensor of E–C coupling [4]. Although the exact signal transduction mechanism between DHPR and RyR is still unknown, it is generally accepted that intramembrane charge movements and associated conformational changes of DHPR couple depolarisation of the T-tubules and Ca\textsuperscript{2+} release from the SR without entry of external Ca\textsuperscript{2+} through DHPR [5–8]. In E–C coupling of skeletal muscle the role of extracellular Ca\textsuperscript{2+} is limited in maintaining the voltage sensors activatable by occupancy of a ‘priming’ site [5]. In contrast, E–C coupling of cardiac muscle depends on Ca\textsuperscript{2+} entry through sarcolemmal DHPR which triggers Ca\textsuperscript{2+} release from SR [9].

Cultured human skeletal muscle cells are often used as a model to study muscle pathology in which Ca\textsuperscript{2+} homeostasis might be disturbed, e.g. Duchenne muscular dystrophy [10–12] and myotonic dystrophy [13]. However, the E–C coupling mechanism of these muscle cells was not examined. Moreover, Cognard and co-workers [14,15] demonstrated a co-existence of the ‘cardiac’ and ‘skeletal’ type of E–C coupling in cultured rat myotubes during myogenesis. Thereby, they showed that the relative part of Ca\textsuperscript{2+} transients and contractures depending on extracellular Ca\textsuperscript{2+} progressively decreases with the age of the muscle cells.

In this study we demonstrate a ‘skeletal muscle type’ of E–C coupling in cultured human skeletal muscle cells in two different ways. Firstly, the Ca\textsuperscript{2+} responses during prolonged activation of the AChR, TTXR, DHPR or RyR provoked by the specific stimuli ACh, veratridine, high (125 mM) KCl and caffeine, respectively, are equal in the presence and absence of extracellular Ca\textsuperscript{2+}. Secondly, all these Ca\textsuperscript{2+} transients become prevented by inhibition of the RyR using ryanodine. Moreover, the cascade of these Ca\textsuperscript{2+} transients becomes prevented by inhibition of the basal cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{j}) accompanied with a decline of the amplitude of the subsequent Ca\textsuperscript{2+} response, revealing a reduced excitability. This phenomenon is also seen during muscle fatigue. However, muscle fatigue can be due to a reduced SR Ca\textsuperscript{2+} content, changes of intracellular metabolites, particularly an increase of P, concentration and a decrease of pH, and a reduced Ca\textsuperscript{2+} sensitivity of the myofilaments [1]. In the present study, the decline of excitability upon iterative stimulation does not appear to be caused by a depletion of the SR Ca\textsuperscript{2+} content or changes of intracellular pH (pH).

**MATERIALS AND METHODS**

**Materials**

The acetoxymethyl esters of Fura-2 (Fura-2/AM) and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF/AM) were purchased from Molecular Probes, Eugene, OR, USA; acetylcholine chloride (ACh), α-bungarotoxin, veratridine, tetrodotoxin (TTX), caffeine, ryanodine, ionomycin, carbonylcyanide m-chlorophenylhydrazone (CCCP), valinomycin and nigericin were from Sigma, St Louis, MO, USA; and nifedipine was from Bayer, Leverkusen, Germany. Other materials were obtained as described [16,17].

**Human skeletal muscle cell cultures**

Muscle biopsies from m. quadriceps, m. biceps or m. rectus abdominis were obtained from individuals without any known muscular disorder, as approved by the Committee on Medical Ethics of the University of Nijmegen. Samples were dissociated and the isolated satellite cells were cultured on glass coverslips (10 × 30 mm) in serum-containing media [16,17]. All experiments were performed with myotubes obtained after 7–10 days of differentiation.

\[\text{[Ca}^{2+}]_{j}\text{, and pH; on-line ratio measurement and calibration}\]

The free cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{j}) and pH\textsubscript{i} were determined in skeletal muscle cells using Fura–2 [16,17] and BCECF [18], respectively. Briefly, myotubes were loaded with either 5 μM Fura–2/AM or 2.5 μM BCECF/AM for 60–90 min at 37°C in physiological salt solution (PSS; containing in mM: 125 NaCl, 10 NaHCO\textsubscript{3}, 1 NaH\textsubscript{2}PO\textsubscript{4}, 5 KCl, 2 MgSO\textsubscript{4}, 1.8 CaCl\textsubscript{2}, 10 HEPES and 10 glucose; pH 7.4). On-line ratio measurements were recorded with a Shimadzu RF-5000 spectrofluorometer. Fura–2 fluorescence was detected at an emission wavelength of 492 nm (bandwidth 5 nm) and alternating excitation wavelengths of 340 and 380 nm (bandwidth 3 nm). BCECF emission was registrated at 525 nm (bandwidth 3 nm) and alternating excitation wavelengths of 340 and 380 nm (bandwidth 1.5 nm). During the measurements the cells were superfused with PSS (4.0 ml/min; 37°C) with additions as indicated in the results. All fluorescence signals were corrected for autofluorescence. The 340/380 ratios (Fura–2) were calibrated with PSS containing 4 μM ionomycin and 10 mM Ca\textsuperscript{2+} (pH 7.7;
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Using the mono-exponential Equations 2 and 3, respectively:

\[
[Ca^{2+}]_{(n)} = [Ca^{2+}]_{(basal(n-1))} + A \times (1 - e^{(-t/t_d)}) 
\]

Eq. 2

\[
[Ca^{2+}]_{(n)} = [Ca^{2+}]_{(basal(n))} + B \times e^{(-t/t_d)} 
\]

Eq. 3

The constants A and B are:

\[
A = \frac{1 - e^{(-t_{max} - t_{min})}}{t_{max} - t_{min}} \times ([Ca^{2+}]_{(max(n))} - [Ca^{2+}]_{(basal(n-1))}) 
\]

\[
B = \frac{([Ca^{2+}]_{(max(n))} - [Ca^{2+}]_{(basal(n))})}{t_{max} - t_{min}} 
\]

in which \( t_s \) and \( t_{max} \) are the times when a stimulus is added or a stimulus-evoked maximal Ca\(^{2+}\) concentration is reached. \( [Ca^{2+}]_{(max)} \) and \( [Ca^{2+}]_{(basal)} \) represent the amplitude of a Ca\(^{2+}\) response and the basal \( [Ca^{2+}] \), at the end of a repolarisation interval, respectively. The stimulus number is given by \( n \). In the case of a single prolonged stimulation (\( n = 1 \)), \( [Ca^{2+}]_{(basal(0))} \) is the resting \( [Ca^{2+}]_r \).

**Statistics**

Data represent means ± SD. Statistical analysis was performed by means of the unpaired Student's t-test and significance was set at \( P < 0.01 \). Curve fittings were obtained by (non-)linear regression analysis using Fig.P® (Biosoft, Cambridge, UK).

**RESULTS**

**Calibration of \([Ca^{2+}]_r\), the resting \([Ca^{2+}]_r\), and the effect of extracellular \(Ca^{2+}\) omission**

The calibration parameters \( R_{max} \) and \( R_{min} \) of Equation 1 are set by the \( Ca^{2+}\) ionophore ionomycin in the presence of an excess of extracellular \( Ca^{2+}\) and subsequently EGTA. The means ± SD are 4.05 ± 0.27 (\( n = 43 \)) and 1.11 ± 0.04 (\( n = 41 \)), respectively. The constant 3, i.e. the ratio of the fluorescence emission of the free dye and the \( Ca^{2+}\)-saturated dye measured at 380 nm, is 1.98 ± 0.09 (\( n = 41 \)). The resting \([Ca^{2+}]_r\) in cultured skeletal muscle cells of controls is 135 ± 12 nM (\( n = 82 \)). Omission of extracellular \( Ca^{2+}\), by superfusion with 1 mM EGTA containing \( Ca^{2+}\)-free PSS, does not affect the resting \([Ca^{2+}]_r\), for at least 5 min. When the effect of a stimulus in the absence of extracellular \( Ca^{2+}\) is studied, superfusion with the EGTA-containing \( Ca^{2+}\)-free PSS precedes the stimulus for 3 min.

**Calibration of \(pH\), and the resting \(pH\)**

Calibration of \(pH\) is performed using high K\(^+\) buffers of various \(pH\) (6.75–8.00) containing nigericin, the K\(^+\)
ionophore valinomycin, the H+ ionophore CCCP and the metabolic inhibitors KCN and iodoacetic acid. The ratio of the BCECF fluorescence and pH are linearly related between pH 6.75 and 8.00. The resting pH, is 7.23 ± 0.04 (n = 6).

The effects of acetylcholine, veratridine, high K+ and caffeine on [Ca2+].

ACh induces a dose-dependent increase of [Ca2+]i. The Ca2+ response is maximal at 20 μM ACh and is independent of extracellular Ca2+ (Fig. 1A–C). The half-maximal effective concentration (EC50) of ACh is approximately 8 μM in the presence of external Ca2+ (Fig. 1B). Inhibition of the AChR by 5 μM α-bungarotoxin completely prevents the ACh-induced Ca2+ transient (Fig. 1D). Tetrodotoxin (5 μM), which inhibits TTX-sensitive voltage-operated Na+ channels, suppresses the ACh-provoked Ca2+ response by 55 ± 6% (n = 5), whereas both nifedipine (5 μM) and ryanodine (10 μM), i.e. specific inhibitors of the voltage sensor and SR Ca2+ release channel, respectively, completely abolish the ACh-induced rise of [Ca2+]i.

Veratridine, an agonist of voltage-operated Na+ channels, raises [Ca2+]i dose-dependently. A maximal Ca2+ response is elicited by 40 μM veratridine in the presence as well as absence of extracellular Ca2+ (Fig. 2A–C). The EC50 of veratridine is circa 25 μM (Fig. 2B). TTX (5 μM) inhibits the veratridine-induced [Ca2+]i increase by 57 ± 5% (n = 7) (Fig. 2D). α-Bungarotoxin does not affect the increase of [Ca2+]i caused by veratridine, in contrast to nifedipine as well as ryanodine which totally suppress this Ca2+ response.

Application of high K+ solutions, prepared by equimolar replacement of NaCl by KCl in PSS, dose-dependently elevates [Ca2+]i. The Ca2+ response is maximal at 125 mM K+ and does not depend on extracellular Ca2+ (Fig. 3A–C). The EC50 of KCl is about 60 mM (Fig. 3B). The K+-induced Ca2+ transient is prevented by nifedipine or ryanodine, but not by TTX (Fig. 3D).

Caffeine generates a dose-dependent Ca2+ response, which is maximal at 40 mM and independent of extracellular Ca2+ (Fig. 4A–C). The EC50 of caffeine is approximately 25 mM (Fig. 4B). Ryanodine (10 μM), unlike nifedipine, inhibits the caffeine-triggered Ca2+ transient (Fig. 4D).

| Table 1 Ca2+ transients induced by acetylcholine, veratridine, KCl or caffeine in the presence or absence of extracellular Ca2+ |
|-----------------|----------------|----------------|----------------|----------------|
| [Ca2+]o (mM)   | Parameter      | ACh (20 μM)    | Veratridine (40 μM) | KCl (125 mM)  | Caffeine (40 mM) |
| 1.8            | [Ca2+]i,0     | 1098 ± 243     | 1006 ± 291         | 1205 ± 171    | 1545 ± 214       |
|                | τi             | 16 ± 4         | 16 ± 5             | 15 ± 5        | 20 ± 7           |
|                | τo             | 23 ± 5         | 25 ± 7             | 23 ± 6        | 29 ± 8           |
|                | (29)           | (9)            | (8)               | (53)          | (11)             |
| 0              | [Ca2+]i,0     | 997 ± 319      | 1001 ± 206         | 1132 ± 298    | 1563 ± 273       |
|                | τi             | 15 ± 6         | 15 ± 5             | 15 ± 5        | 20 ± 6           |
|                | τo             | 22 ± 5         | 23 ± 6             | 21 ± 7        | 28 ± 8           |
|                | (9)            | (5)            | (5)               | (15)          | (6)              |

Values are means ± SD of the number of individual cultures listed between parentheses. The amplitude of the Ca2+ responses ([Ca2+]i,0) is expressed in nM and the halftime of the increasing (τi) or decaying phases (τo) in s. The resting [Ca2+]i in the presence or absence of external Ca2+ is 135 ± 12 nM (82) and 129 ± 16 nM (31), respectively.
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Ca\(^{2+}\) as well as the half-increase and half-decay times is given in Table 1. The effects of the inhibitors α-bungarotoxin, tetrodotoxin, nifedipine or ryanodine on these Ca\(^{2+}\) transients are listed in Table 2.

The effects of repetitive 125 mM K\(^+\) stimulation on [Ca\(^{2+}\)], and pH

To study the excitability of cultured human skeletal muscle cells, we tried to stimulate the myotubes iteratively using electrical field depolarisations. However, small pulses (10 V) failed to trigger significant Ca\(^{2+}\) transients, whereas larger pulses (25–100 V) induced electroporation of the sarcolemma. Only 125 mM K\(^+\) was useful to perform repetitive stimulation. ACh as well as veratridine and caffeine require very long repolarisation times (5–10 min) before a second Ca\(^{2+}\) transient can be elicited (data not shown), although the half-decay times of the Ca\(^{2+}\) responses to those agents are similar to that of high K\(^+\) (Table 1).

The muscle cells were 10 times iteratively depolarized by superfusion with 125 mM K\(^+\) for 60 s and subsequently repolarized for 180, 120, 60 or 30 s with PSS. During repeated K\(^+\) stimulation, the characteristics of a Ca\(^{2+}\) response, [Ca\(^{2+}\)]\(_{\text{IMax}}\) and [Ca\(^{2+}\)]\(_{\text{IBasal}}\), \(\tau_i\) and \(\tau_d\) appear to depend on the repolarisation interval between successive K\(^+\) stimulations. Already at the lowest stimulus frequency, i.e. 180 s repolarisation time between two stimuli, the excitability of the muscle cells declines (Fig. 5A). Thereby [Ca\(^{2+}\)]\(_{\text{IMax}}\) decreases to 67% of the initial amplitude, whereas [Ca\(^{2+}\)]\(_{\text{IBasal}}\) increases to 140% of the resting [Ca\(^{2+}\)]\(_i\), and \(\tau_i\) as well as \(\tau_d\) rise to 126% of their original values. When the repolarisation time is shortened, the Ca\(^{2+}\) transients become even smaller and slower (Fig. 5B–D). The raise of [Ca\(^{2+}\)]\(_{\text{IBasal}}\), the decline of [Ca\(^{2+}\)]\(_{\text{IMax}}\) and the increase of both \(\tau_i\) and \(\tau_d\) become enlarged (Fig. 6). However, when [Ca\(^{2+}\)]\(_{\text{IBasal}}\) equals the resting [Ca\(^{2+}\)]\(_i\), i.e.

Table 2 Ca\(^{2+}\) transients induced by acetylcholine, veratridine, KCl or caffeine in the presence of specific inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ACh (20 \text{nM})</th>
<th>Veratridine (40 \text{nM})</th>
<th>KCl (125 mM)</th>
<th>Caffeine (40 \text{mM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1098 ± 243</td>
<td>1006 ± 291</td>
<td>1205 ± 171</td>
<td>1545 ± 214</td>
</tr>
<tr>
<td>α-Bungarotoxin</td>
<td>138 ± 21</td>
<td>1045 ± 260</td>
<td>n.d.</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>(5 \text{μM})</td>
<td>(7)</td>
<td>(4)</td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>529 ± 118</td>
<td>519 ± 107</td>
<td>1228 ± 210</td>
<td>n.d.</td>
</tr>
<tr>
<td>(5 \text{μM})</td>
<td>(5)</td>
<td>(7)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>130 ± 18</td>
<td>126 ± 30</td>
<td>133 ± 21</td>
<td>1514 ± 266</td>
</tr>
<tr>
<td>(5 \text{μM})</td>
<td>(12)</td>
<td>(7)</td>
<td>(14)</td>
<td>(4)</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>131 ± 29</td>
<td>139 ± 24</td>
<td>128 ± 32</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>(10 \text{μM})</td>
<td>(12)</td>
<td>(4)</td>
<td>(14)</td>
<td>(6)</td>
</tr>
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</table>

Values given in nM are means ± SD of the number of individual cultures listed between parentheses. n.d., not determined.
within 600 s repolarisation, the initial $\text{Ca}^{2+}$ response is recovered (Fig. 5C,D).

The decrease of $[\text{Ca}^{2+}]_{\text{max}}$ is not related to depletion of the intracellular $\text{Ca}^{2+}$ store, i.e. SR, since addition of 40 mM caffeine immediately after the tenth $K^+$ exposure of each stimulation protocol reveals a normal $\text{Ca}^{2+}$ response ($1.47 \pm 0.20 \mu M$ ($n = 12$); Fig. 7). A change of $\text{pH}_i$ does not explain the reduction of $[\text{Ca}^{2+}]_{\text{max}}$ too, because the $\text{pH}_i$ is not affected in our cultured muscle cells during iterative $K^+$ stimulation, whatever the repolarisation time was (Fig. 5).

In search of a $[\text{Ca}^{2+}]_{\text{i}}$-regulated fine-tuning of the $\text{Ca}^{2+}$ responses during repetitive $K^+$ stimulation, we evaluated the correlations between the various $\text{Ca}^{2+}$ response characteristics of the increasing and decaying phase, respectively. For that purpose the values from all iterative stimulation experiments are taken into account. $[\text{Ca}^{2+}]_{\text{in}}$ inversely correlates with $[\text{Ca}^{2+}]_{\text{inax}}$ and exhibits a high correlation with $\tau_i$ (Fig. 8A,B). Besides, an inverse correlation exists between $[\text{Ca}^{2+}]_{\text{inax}}$ and $\tau_d$ (Fig. 8C).

**DISCUSSION**

In skeletal muscle of vertebrates, E–C coupling is thought to occur by a 'mechanical coupling', in which the T-tubular voltage-sensing DHPR activates the SR RyR. In a subsequent step, the released $\text{Ca}^{2+}$ amplifies the SR $\text{Ca}^{2+}$ release by activating RyRs that are not linked to DHPRs [7,20]. Additionally, skeletal muscle specific isoforms of both DHPR and RyR are required for a skeletal muscle E–C coupling [21,22]. However, our understanding of E–C coupling has almost exclusively been derived from experiments on adult muscle fibres from frogs, mice and rats. Recently, fundamental differences have been observed in control mechanisms of E–C coupling in skeletal muscle of amphibians and rodents [23], as well as humans [24].

Although it is possible to examine ion homeostasis in cut or resealed human muscle fibres [24,25], the use of these muscle preparations has several disadvantages. Muscle fibres have a short lifetime in vitro and do not retain their properties in culture, due to dedifferentiation. Therefore, it is impossible to perform extensive studies on isolated fibres. Furthermore, preparation of single muscle fibres might result in the lost of cytosolic compounds, which are important for ion homeostasis and energy metabolism. Isolated mononuclear satellite cells are also unsuitable because these undifferentiated muscle cell precursors lack a signal transduction system which resembles the in vivo situation [15]. Cultured human myotubes have often been used to investigate muscle pathology, in which ion homeostasis is affected, but their E–C coupling mechanism is poorly understood.

The resting $[\text{Ca}^{2+}]_{\text{i}}$, and the effect of external $\text{Ca}^{2+}$ omission

The resting $[\text{Ca}^{2+}]_{\text{i}}$ values of muscle cells presented in the literature vary markedly and seem to depend on the cell origin, the $\text{Ca}^{2+}$-indicator and the calibration procedure applied. Using the in vivo calibration of Fura–2, the currently estimated basal $[\text{Ca}^{2+}]_{\text{i}}$ agrees with published data of cultured human myotubes [10,13,16,17,26] and myofibers in vivo [25]. The resting $[\text{Ca}^{2+}]_{\text{i}}$ is not changed by superfusion with 1 mM EGTA containing $\text{Ca}^{2+}$-free PSS for 3 min. Since all stimuli-induced $\text{Ca}^{2+}$ responses in the absence of external $\text{Ca}^{2+}$ are equal to those measured in the presence of $\text{Ca}^{2+}$, it can be concluded that neither does the voltage sensor become 'deprimed' nor is the intracellular $\text{Ca}^{2+}$ store depleted by the omission of extracellular $\text{Ca}^{2+}$. At longer periods of external $\text{Ca}^{2+}$ deprivation and/or higher concentrations of EGTA, progressive inactivation of the DHPR, or a $\text{Ca}^{2+}$ leakage as well, may occur [5,15].
The type of E–C coupling mechanism

To elucidate the type of E–C coupling in cultured human skeletal muscle cells, we investigated the effect of membrane depolarization on Ca2+ homeostasis in the presence as well as absence of external Ca2+. Depolarization of the sarcolemma was achieved in three different ways: first, by ACh-induced opening of AChRs [13]; second, by veratridine-triggered activation of voltage-operated Na+-channels [27]; and third, by changing the K+ gradient [15]. Additionally, Ca2+ releasable from SR was examined by caffeine stimulation. Since the amplitudes of the Ca2+ responses during prolonged excitation with ACh, veratridine, KCl or caffeine are equal both in the presence and absence of extracellular Ca2+ (Table 1), the rise of [Ca2+]i has to originate from the Ca2+ release of the SR. This indicates a 'skeletal muscle type' of E–C coupling in the cultured human skeletal muscle cells rather than a 'cardiac muscle type', in which a Ca2+-induced Ca2+ release mechanism causes the signal transduction [14]. Our result is in accordance with the recent finding of a skeletal muscle type coupling between excitation and Ca2+ transient induced in human myotubes at electrical stimulation [28]. However, much smaller Ca2+ responses (100 nM) were observed than the agonist-generated Ca2+ transients in the present study (~ 1 μM).

The E–C coupling cascade

For examination of the cascade of events involved in E–C coupling of human myotubes, we studied the effects of the above mentioned stimuli on Ca2+ homeostasis after selective inhibition of an ion channel or receptor. Ca2+ transients induced by ACh, veratridine, 125 mM K+ and caffeine are mediated through activation of AChR, TTXR, DHPR and RyR, respectively as attested by the blocking effects of α-bungarotoxin, TTX, nifedipine or ryanodine on the appropriate stimulus-induced Ca2+ responses. The partial inhibition of Ca2+ responses by tetrodotoxin agrees with the coexistence of TTX-sensitive and TTX-resistant voltage-operated Na+ channels in cultured skeletal muscle cells [27]. It should be noted that electrochemical depolarization of the sarcolemma using K+ concentrations lower than 125 mM, i.e. Na+ concentrations...
Fig. 6 Characteristics of Ca\textsuperscript{2+} transients in cultured muscle cells during repeated 125 mM K\textsuperscript{+} stimulation. [Ca\textsuperscript{2+}]\textsubscript{i, base} (A), [Ca\textsuperscript{2+}]\textsubscript{i, max} (B), \(\tau\) (C) and \(\tau\) (D) during the \(n\)th stimulation at repolarisation intervals of 180 s (open circles), 120 s (triangles), 60 s (squares) or 30 s (inverted triangles). Data result from 21 different muscle cell cultures.

Fig. 7 Caffeine-induced Ca\textsuperscript{2+} responses following iterative 125 mM K\textsuperscript{+} stimulation at repolarisation intervals of 180 s (A), 120 s (B), 60 s (C) or 30 s (D). Application of 40 mM caffeine (marked by the white bars) was performed after the last two K\textsuperscript{+} exposures (marked by the black bars) of each stimulation protocol as shown in Figure 5.

higher than 11 mM, might provoke a Na\textsuperscript{+}-influx as well by activating voltage-operated Na\textsuperscript{+} channels. However, since stimulation of DHPR is prevented by both nifedipine and ryanodine, excitation of TTXR is inhibited by TTX, nifedipine as well as ryanodine, and activation of AChR is suppressed by all inhibitors, it is concluded that the E–C coupling cascade in cultured human muscle cells is mediated by subsequently AChR, voltage-operated Na\textsuperscript{+}-channels, DHPR and RyR. This sequence of events resembles the in vivo situation.

The fact that inhibition of the SR Ca\textsuperscript{2+} release channel by ryanodine prevents depolarization-induced Ca\textsuperscript{2+} transients in human myotubes is another evidence for a 'skeletal muscle type' of E–C coupling in these cells. In
addition, it suggests that, during excitation of the skeletal muscle cells, an influx of external Ca\textsuperscript{2+} through the ion unselective AChR [29], the dihydropyridine-insensitive fast-activated voltage-operated Ca\textsuperscript{2+} channel (T-type) [30] and/or slow-activated voltage-dependent Ca\textsuperscript{2+} channel (DHPR) [3] either may be excluded or is ineffective in inducing Ca\textsuperscript{2+} release from the SR. Ca\textsuperscript{2+} entry via DHPR might become evident at long-lasting strong depolarisation, but it occurs later in time than the charge movement in DHPR and the associated activation of RyR [31].

\textbf{[Ca\textsuperscript{2+}]}\textsuperscript{response kinetics}

The shape of Ca\textsuperscript{2+} transients is defined by two opposite acting mechanisms. On the one hand, in the presence of ACh, veratridine or KCl the sarcolemma is depolarized and the DHPR-RyR complex causes SR Ca\textsuperscript{2+} release. On the other hand, the SR Ca\textsuperscript{2+}-ATPase, which is activated by the increased [Ca\textsuperscript{2+}]{_\text{iv}} counteracts the release. Furthermore, during (prolonged) excitation the AChR, TTXR, DHPR and RyR may become desensitized and/or inactivated [8,32,33].

The half-increase and half-decay times of the stimulus-generated Ca\textsuperscript{2+} transients in human muscle cells cultured on serum are much slower than those in mouse and rat myotubes [15,34–36] or human muscle fibres [24]. This is due to the fact that the serum-derived human myotubes do not reach the same level of differentiation. Maturation of mouse myotubes is accompanied by a uniform rearrangement of initially disordered triads with myofilaments, which results in increased Ca\textsuperscript{2+} release and reuptake rates [37]. A differentiation arrested and immature contractile machinery explains the lack of contractions in serum cultured human myotubes, even after stimulation, as observed by us and others [28,38,39]. Ultroser-cultured human muscle cells attain much higher differentiation levels than serum-cultured cells [40,41]. The Ca\textsuperscript{2+} homeostasis of the highly matured Ultroser-derived human myotubes, however, could not be examined, because these cells detach from the glass coverslips during their final differentiation or upon stimulation, due to contractions. Despite we tested various coatings (collagen I or IV, gelatin, poly-L- or -D-lysine, matrigel) attachment of Ultroser-cultured myotubes could not be improved. Plastic coverslips are neither useful since these introduce strong autofluorescence.

\textbf{Excitability during iterative 125 mM K\textsuperscript{+} stimulation}

ACh, veratridine as well as caffeine require very long repolarisation times before a second Ca\textsuperscript{2+} response can be triggered. This is probably due to a desensitisation of the agonist-binding site of the appropriate ion channel. Repetitive stimulation of cultured human skeletal muscle cells is only effectively performed by 125 mM K\textsuperscript{+}. The amplitude of a Ca\textsuperscript{2+} transient elicited during repetitive K\textsuperscript{+} stimulation appeared to depend on the [Ca\textsuperscript{2+}], prior to stimulation, which on its turn seems to depend on the repolarisation interval between successive stimulations. At shorter repolarisation times an increase of the basal [Ca\textsuperscript{2+}], is followed by a decrease of the amplitude of the subsequent Ca\textsuperscript{2+} response, indicating a reduced excitability. A similar phenomenon is seen during fatigue in frog and mouse muscle fibres [1,42]. However, muscle fatigue is a complex phenomenon, in which multiple factors are involved as a decreased Ca\textsuperscript{2+} content of the SR, reduction
of the Ca\(^{2+}\) release from the SR, a decreased Ca\(^{2+}\) reuptake, changes in pH, and/or metabolite (ATP, PCr, lactate) or electrolyte ([K\(^+\)]\(_i\)) concentrations and a reduced Ca\(^{2+}\) sensitivity of the myofilaments [1].

The loss of excitability in our cells does not result from a Ca\(^{2+}\) depletion of the SR as confirmed by a normal caffeine-induced Ca\(^{2+}\) response immediately after the tenth K\(^+\) stimulation of each stimulation protocol. In our experimental setup, an elevation of the extracellular K\(^+\) content can be ruled out, because of a continuous wash-out by superfusing PSS during repolarisation. Acidosis of skeletal muscle has been shown to reduce both SR Ca\(^{2+}\) release and reuptake [42]. However, we did not detect any change of the pH\(_i\) during iterative K\(^+\) stimulation (Fig. 5). Data of resting pH\(_i\) in cultured human muscle cells are not available in the literature. The presently measured value (7.23) is comparable to values from skeletal muscle of mice and humans [43,44]. The concomitant increase of basal [Ca\(^{2+}\)]\(_i\) and decrease of Ca\(^{2+}\) response suggest a [Ca\(^{2+}\)]\(_i\)-regulated fine-tuning of the SR Ca\(^{2+}\) release during repeated K\(^+\) stimulation. This is in accordance with Ca\(^{2+}\)-dependent feedback mechanisms, known to control the DHPR-RyR complex [78,45]. Thereby Ca\(^{2+}\) depletion of the SR is prevented and the excitation threshold is increased to exclude muscle damage [42]. Our observations also agree with the indication that alterations in intracellular Ca\(^{2+}\) exchange play a major role in muscle fatigue [46].

In conclusion, cultured human skeletal muscle cells possess a 'skeletal muscle type' of E-C coupling and exhibit a strong dependence between the basal [Ca\(^{2+}\)]\(_i\) and the Ca\(^{2+}\) transient amplitude upon stimulation. These findings make cultured human skeletal muscle cells highly suitable for studying ion homeostasis in relation to muscle pathology or muscle fatigue.

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