The Creatine Kinase System Is Essential for Optimal Refill of the Sarcoplasmic Reticulum Ca\(^{2+}\) Store in Skeletal Muscle*  

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Muscle function depends on an adequate ATP supply to sustain the energy consumption associated with Ca\(^{2+}\) cycling and actomyosin sliding during contraction. In this regulation of energy homeostasis, the creatine kinase (CK) circuit for high energy phosphoryl transfer between ATP and phosphocreatine plays an important role. We earlier established a functional connection between the activity of the CK system and Ca\(^{2+}\) homeostasis during depolarization and contractile activity of muscle. Here, we show how CK activity is coupled to the kinetics of spontaneous and electrically induced Ca\(^{2+}\) transients in the sarcoplasm of myotubes. Using the UV ratiometric Ca\(^{2+}\) probe Indo-1 and video-rate confocal microscopy in CK-proficient and -deficient cultured cells, we found that spontaneous and electrically induced transients were dependent on ryanodine-sensitive Ca\(^{2+}\) release channels, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase pumps, extracellular calcium, and functional mitochondria in both cell types. However, at increasing sarcoplasmic Ca\(^{2+}\) load (induced by electrical stimulation at 0.1, 1, and 10 Hz), the Ca\(^{2+}\) removal rate and the amount of Ca\(^{2+}\) released per transient were gradually reduced in CK-deficient (but not wild-type) myotubes. We conclude that the CK/phosphocreatine circuit is essential for efficient delivery of ATP to the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase pumps and thereby directly influences sarcoplasmic reticular refilling and the kinetics of the sarcoplasmic Ca\(^{2+}\) signals.

Ionic Ca\(^{2+}\) regulates numerous cellular processes such as contraction, synaptic transmission, gene expression, metabolism, and cell death (1). In skeletal muscle, Ca\(^{2+}\) is a key regulator of contractile activity and glycogenolytic (activation of phosphorylase) and mitochondrial (activation of Ca\(^{2+}\)-sensitive dehydrogenases) ATP production (2, 3). This is especially important during cycles of repetitive muscle contractions, when myosin ATPase activity is high and large Ca\(^{2+}\) loads have to be actively extruded from the cytosol by the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and/or plasma membrane Ca\(^{2+}\)-ATPase.

To achieve robust buffering of levels of cellular ATP and to optimize its transport between the sites of production and consumption by ATPases, excitable tissues such as muscle and brain utilize high energy phosphoryl transfer and ATP-regenerating pathways such as the creatine kinase (CK; EC 2.7.3.2) and adenylyl kinase (EC 2.7.4.3) systems (4–6). In adult muscle, two CK subunits are expressed: cytosolic M-CK, which is active as a muscle-muscle isoform dimer, and ScCKmit, which is present as inactive homodimeric and active homo-oligomeric molecules in the mitochondrial intermembrane space. In newborn or embryonic muscle, expression of either BB- or BM-CK is seen, but expression of these proteins gradually decreases when the muscle matures. The presence of CK at the sarcomeric M- and I-bands and at the sarcoplasmic reticulum (SR) membrane is important for regenerating adequate amounts of ATP for the myosin ATPase (contraction) and the SERCA pump (Ca\(^{2+}\) homeostasis) (7, 8). CK in mitochondrial protein complexes, together with the voltage-dependent anion channel and the adenine nucleotide transporter, is important in regulating intramitochondrial ATP/ADP ratios (9). In previous studies, we observed that cellular calcium homeostasis in primary cultures of CK-deficient myotubes (referred to as CK\(^{-/-}\); M-CK\(^{-/-}\) × ScCKmit\(^{-/-}\)) is abnormal. It has been speculated that these alterations could be reversible for the anomalously contractile properties of these muscles in vivo (10). Apart from the changes in contractile properties, we also observed extensive remodeling of muscle ultrastructure and a rewiring of fluxes through metabolic pathways as a response to gene deficiency (10–15). Given the effects of local [Ca\(^{2+}\)] on metabolic activity (3, 16–18) and its importance as a general signaling molecule in the highly plastic muscle cell (19), the ultrastructural and physiological adaptation in our CK-deficient mice may (at least in part) result from changes in Ca\(^{2+}\) signaling (1, 19, 20). Indeed, various studies point to a direct involvement of CK in optimization of SERCA function and calcium homeostasis in skeletal muscle (8, 21–23), suggesting a direct link between regulation of appropriate (local) ATP/ADP ratios and Ca\(^{2+}\) handling. To obtain a better understanding of this connection and its coupling to different Ca\(^{2+}\) signaling pathways, we need a more detailed picture of amplitude and frequency behavior of

* This work was supported by Program Grant 901-01-095 from the Dutch Organization for Scientific Research (Medical Sciences). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase; CK, creatine kinase; SR, sarcoplasmic reticulum; [Ca\(^{2+}\)], sarcoplasmic Ca\(^{2+}\) concentration; ROI, region of interest; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
Ca²⁺ transients in CK-deficient muscles. Here, we studied mechanistic and kinetic aspects of Ca²⁺ homeostasis in immortalized myotubes using the UV ratiometric probe Indo-1 and video-rate confocal microscopy. CK-proficient and -deficient immortalized cell lines were derived from wild-type and CK⁻/⁻ mice crossed to H-2Kᵇ-tsA58 transgenic mice (24) and differentiated in vitro. By analysis of spontaneous and electrically induced sarcoplasmic Ca²⁺ transients, we found that in contrast to wild-type myotubes, CK-deficient myotubes displayed a gradual reduction of the Ca²⁺ removal rate and SR refilling with increasing stimulation intensity. We conclude that the CK/phosphocreatine circuit is essential for efficient SERCA-mediated refilling of the SR and thus for the shaping of sarcoplasmic Ca²⁺ signals.

MATERIALS AND METHODS

Culture of Immortalized Cell Lines—The generation of immortalized cell lines, the growth of myotubes, and culture conditions were as described by Morgan et al. (26). We derived immortalized cell lines from wild-type and CK⁻/⁻ mice. Myoblasts were cultured at low density at permissive temperature (33 °C, 10% CO₂) in Dulbecco’s modified Eagle’s medium (Invitrogen, Breda, The Netherlands) supplemented with 20% fetal calf serum (Integro, Zaandam, The Netherlands), 2% chicken embryo extract (Invitrogen), and 20 units/ml murine recombinant interferon-γ (Invitrogen). For differentiation at nonpermissive temperature (37 °C, 5% CO₂), cells were cultured in a high density Matrigel (Becton Dickinson, Heidelberg, Germany)-coated plastic Aclar coverslips (Allied-Signal, Pottsville, PA) in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum (Invitrogen) in the presence of 20 mM creatine (Sigma, Zwijndrecht, The Netherlands), which improves myoblast fusion characteristics in both wild-type and CK-deficient cell lines.

Confocal Calcium Measurements—Myotubes (at 5 days of differentiation) were washed with physiological salt solution (125 mM NaCl, 10 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM KCl, 2 mM MgSO₄, 1.8 mM CaCl₂, and 10 mM glucose, pH 7.4). For Ca²⁺ recordings, myotubes were exposed for 30 min at 37 °C to 10 μM Indo-1/AM in the presence of 0.025% (w/v) Pluronic F-127 (Molecular Probes, Inc., Leiden, The Netherlands) in physiological salt solution, washed twice, and allowed to equilibrate for 15 min. Use of the ratiometric Ca²⁺ probe Indo-1 in myotubes has been described earlier (27, 28) and largely circumvents problems associated with non-ratiometric Ca²⁺ probes (like dye compartmentalization, leakage, and photobleaching). After an additional wash to remove residual traces of Indo-1/AM, coverslips were put in a Leiden chamber (volume of 700 μl) and placed on a Nikon Diaphot inverted microscope attached to an OZ confocal laser scanning microscope (Noran Instruments, Naarden, The Netherlands). Indo-1 was excited at 351 nm using a modified high power argon ion laser (Coherent Enterprise, Santa Clara, CA). Indo-1 emission light was collected using a Nikon ×40 water immersion objective (NA1.2) with high UV transmission, separated by a 455 DCLP dichroic mirror, and quantified at 405 ± 45 and 485 ± 45 nm using photomultipliers. To increase the signal-to-noise ratio, no confocal slit was used. This maximized the confocal detection volume (section thickness of ~5–8 μm) (30) and enabled us to run the laser at minimal levels (output of 15 milliwatts, equivalent to 28 microwatts at the back of the objective lens).

Basil ratio signals were stable, and no UV-induced deterioration in cell viability was observed during experiments. In long experiments (>5 min), the UV laser and recordings were stopped between measurements to prevent UV damage. The OZ hardware was controlled by Intervision Version 1.5 acquisition software (Noran Instruments) running under IRIX 6.5 on an Indy workstation (Silicon Graphics Inc., Mountain View, CA) equipped with 256-MB internal memory. Before recording images, a custom memory-management script was used to ensure optimal timing efficiency. When image storage was not required, raw Indo-1 ratio signals were visualized in real-time using a software plotting application and successively stored in ASCII format. Emission signals were separately background-corrected before calculating ratio signals (405/485 nm).

Superfusion and Electrical Stimulation—Inhibitors (Sigma) were either directly dissolved in physiological salt solution or diluted from a concentrated stock solution in Me₂SO and used at the concentrations indicated (final Me₂SO concentrations never exceeded 0.1% (v/v)). Chemical depolarization was performed using physiological salt solution containing 125 mM KCl. To generate Ca²⁺-free medium, CaCl₂ was omitted, and 500 μM EGTA was added. All solutions were administered by a Leiden chamber using a custom-made superfusion setup (1.5 ml/min), except for thapsigargin (pipetted). Temperature was held constant by a TC-102 temperature controller (Medical Systems Corp., Greensvale, NY). For electrical stimulation, two platinum electrodes were fixed to both sides of the chamber and connected to a Grass SD9 stimulator. Myotubes were stimulated (16 V over 10 ms) at frequencies indicated below.

Data Analysis—For quantitative analysis, ratio signals were normalized relative to the basal resting Indo-1 ratio. We used arbitrary units for these normalized Indo-1 ratios (30, 31). Off-line analysis of numerical data was performed with Origin Pro 6.1 (Microcal, Northampton, MA). Images were analyzed using Noran 2D software (Noran Instruments) and Image Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD). Numerical data obtained from multiple recordings are expressed as mean ± S.E. unless stated otherwise. Statistical significance (tested using a t test) is expressed as p < 0.05, p < 0.01, or p < 0.001. During curve fitting, we used Pearson’s correlation coefficient R (or product moment correlation coefficient) as an estimate of the population correlation coefficient. Values of −1 and +1 indicate a perfect linear relationship between the two variables. The coefficient of determination (R²) was used to express the proportion of the total variation that is explained by the regression. In analogy to R, if R² = 1, the total variation in the y-variable can be explained in terms of the regression curve.

RESULTS

Spontaneous Contractions and [Ca²⁺], Transients in Myotubes—After 3–5 days of differentiation, both wild-type and CK-deficient (CK⁻/⁻) myotubes displayed periods of spontaneous contractions in culture, separated by silent periods. Video-rate (30 Hz) confocal microscopy of myotubes loaded with Indo-1 revealed, as anticipated, that these contractions were paralleled by transient rises in the [Ca²⁺], transients. At the subcellular level, these [Ca²⁺], transients appeared as unidirectional waves with a sharp front. Fig. IA displays a typical example of such a wave recorded from a wild-type myotube (but this figure is representative of both cell types). Three consecutive Indo-1 ratio images (each 479 × 512 pixels, corresponding to 90.3 × 96.6 μm) taken from a 5-s recording are shown. For optimal visualization, the images taken at both Indo-1 emission wavelengths were first de-blurred using deconvolution software (Noran 2D analysis) and then background-corrected before ratios were calculated. Next, the resulting Indo-1 ratio signal was height-coded after color conversion using a linear look-up table. Analysis of the figure revealed that the [Ca²⁺], wave progressed about 550 pixels (≈100 μm) within 100 ms, roughly equating a wave speed of 1000 μm/s (experimental performed at room temperature).

The change in average [Ca²⁺], was investigated by analyzing a region of interest (ROI) that encompassed the entire myotube. [Ca²⁺], reached its maximum within 99 ms (three data points obtained from the images in Fig. IA), after which it rapidly declined to basal levels (Fig. IB). This [Ca²⁺], decline was adequately described (in Fig. IB, R² was 0.997) by a monoexponential function (Y = Y₀ + Ce⁻μt), suggesting the involvement of one major removal process described by a single rate constant μ (31). We used this rate constant, which is inversely proportional to the rate of [Ca²⁺], decline, as a quantitative readout of the sarcoplasmic Ca²⁺ removal kinetics. For fitting the [Ca²⁺], decline, we applied the robust Levenberg-Marquardt algorithm (32). In the trace shown, the decay constant equaled 0.220 s. Because the residence time at maximal amplitude was only 33 ms (i.e. one data point), no attempt was made to quantify the amplitude of the [Ca²⁺], transients. When recording full resolution images at 30 Hz, the size of the frame store memory limited total recording time to 5 s. To allow...
longer recordings, we disabled image collection, and stored only numerical data for different sarcoplasmic ROIs (Fig. 1D). It was found that different sarcoplasmic ROIs within a single myotube exhibited identical [Ca^{2+}] kinetics (Fig. 1E). This finding enabled random comparison of sarcoplasmic ROIs between different myotubes. Using this approach, we observed (n = 114, equally distributed between wild-type and CK−/− genotypes) that (i) [Ca^{2+}] transients occurred either continuously or as discrete bursts (Fig. 1C, examples from wild-type myotubes); (ii) the amplitude and frequency of these transients were remarkably constant within the same myotube; and (iii) between different myotubes, the frequency was variable (range...
ing from <0.1 to 10 Hz) and increased with temperature (1.6 ± 0.1 Hz (n = 55) and 3.4 ± 0.1 Hz (n = 59) at 22 and 37 °C, respectively). No differences in distribution of the firing frequencies, firing pattern, and temperature sensitivity were found between wild-type and CK −/− myotubes. During the time course of the measurements (5 min of continuous recording at 30 Hz), no significant change in decay constant was observed (data not shown). This indicates that μ also is a reliable parameter for the quantification of [Ca2+]2, kinetics during prolonged recordings.

**Role of CK, SR, and SR Ca2+-ATPase in Spontaneous [Ca2+]2 Transients**—In mature skeletal muscle, depolarizations of the plasma membrane are sensed by the dihydropyridine receptor in the T-tubule membrane. This protein directly interacts with the ryanodine-sensitive Ca2+ release channel RyR in the SR membrane to promote the release of Ca2+ from the SR into the sarcoplasm (2). SERCA pumps in the SR membrane pump Ca2+ from the sarcoplasm back into the SR (41).

Because the excitation-contraction coupling apparatus of skeletal muscle is known to develop over time (33), we analyzed the source of the Ca2+ during spontaneous activity in wild-type and CK −/− myotubes. For both preparations, a specific block of the SERCA pumps with thapsigargin (2 μM) did not lead to immediate cessation of the spontaneous [Ca2+]2 transients. Indeed, it took ~4 min until the spontaneous [Ca2+]2 transients were extinguished (Fig. 2A, representative of 25 experiments in wild-type myotubes). On the other hand, thapsigargin immediately reduced (within 30 s after application) the Ca2+ removal rate and increased the interval between the [Ca2+]2 transients (Fig. 2, B and C). In agreement with this finding, the removal of extracellular Ca2+ did not result in immediate cessation of the spontaneous [Ca2+]2 transients in either wild-type or CK −/− myotubes (Fig. 2D, data from wild-type myotubes, total of eight wild-type and CK −/− genotypes analyzed). Importantly, in the myotube shown (in which the spontaneous [Ca2+]2 transients occurred at a much higher frequency than in Fig. 2A), it took again ~4 min for the [Ca2+]2 transients to extinguish. The spontaneous [Ca2+]2, transients reappeared, although at a somewhat lower frequency, when Ca2+ was re-added to the medium.

To determine whether CK plays a role in the spontaneous [Ca2+]2 transients, we compared the Ca2+ removal kinetics between wild-type and CK −/− myotubes that displayed [Ca2+]2 transients of similar frequencies (3.1 ± 0.3 Hz (n = 23) and 3.6 ± 0.4 Hz (n = 17) for wild-type and CK −/− myotubes, respectively) measured at 37 °C. For analysis, one transient was randomly chosen from each myotube (using random numbers generated with Origin Pro 6.1). Next, the maximal amplitudes of the transients were temporally aligned to allow calculation of the average decline kinetics. Fig. 3 shows that the
Ca^{2+} removal was significantly faster (indicated by the corresponding time points by asterisks) in wild-type myotubes (wild-type, \( \mu = (0.051 \pm 1.8) \times 10^{-3} \) s and \( R^2 = 0.998 \)); and CK\(^{-/-} \), \( \mu = (0.094 \pm 4.6) \times 10^{-3} \) s and \( R^2 = 0.996 \). Taken together, the findings presented in Figs. 2 and 3 suggest that CK is required for an optimal SERCA-mediated refi\( l \) of the SR and that only little Ca\(^{2+} \) is extruded across the plasma membrane during spontaneous [Ca\(^{2+} \)]\(_s\), spiking. Given the results obtained with thapsigargin, we expect that the SR Ca\(^{2+} \) store contains su\( f \)icient Ca\(^{2+} \) to maintain spontaneous Ca\(^{2+} \) release events for prolonged periods of time, even in the absence of functional SERCAs.

Ca\(^{2+} \) Storage Capacity of the SR—To investigate the amount of Ca\(^{2+} \) present in the SR, we performed experiments in which we chemically depolarized wild-type and CK\(^{-/-} \) myotubes by application of an isosmotic medium containing 125 mM KCl (Fig. 4, A–C, wild-type myotubes). This high KCl medium induced a large [Ca\(^{2+} \)]\(_s\), transient that was independent of the presence of extracellular Ca\(^{2+} \) (Fig. 4, compare A and B). When extracellular Ca\(^{2+} \) was removed directly following KCl treatment, a second KCl application failed to induce a [Ca\(^{2+} \)]\(_s\), transient of the same amplitude (Fig. 4B; the area under the second transient (marked II) was reduced by 75%, whereas the duration was similar to that of the first transient (I): 33 s versus 31 s). This indicates that during the first transient, Ca\(^{2+} \) was mainly extruded across the sarcolemma and not re-sequestered into the SR by SERCA pump action. The enlarged inset shows that the rising speed of the small second transient was markedly reduced, whereas the [Ca\(^{2+} \)], decay rate remained virtually unaltered. Fig. 4C shows that the KCl-induced [Ca\(^{2+} \)]\(_s\), transient was fully restored within 10 min following re-addition of Ca\(^{2+} \) to the medium (same myotube as in Fig. 4C). In summary, these data show that high KCl stimulates the extrusion of Ca\(^{2+} \) across the plasma membrane, leading to depletion of the SR Ca\(^{2+} \) store. Therefore, under these conditions, the removal process is entirely dominated by the action of the plasma membrane Ca\(^{2+} \)-ATPase and/or Na\(^+/Ca\(^{2+} \) exchange.

In Fig. 4C, the KCl-induced transient was preceded by spontaneous [Ca\(^{2+} \)]\(_s\), transients. A qualitative comparison of the kinetic characteristics of these two types of [Ca\(^{2+} \)]\(_s\), signal revealed that, in general, the amplitude was lower, the duration was shorter, and the removal rate was more rapid for spontaneous transients. The decline in the KCl-induced [Ca\(^{2+} \)]\(_s\), transient was also monoeponential. In the typical trace shown, the Ca\(^{2+} \) removal rate was \(-200 \) times slower following KCl treatment compared with spontaneous transients, as reflected by an increase in the decay constant from \( 0.2 \pm 0.030 \) s (spontaneous) to \( 38 \pm 1.7 \) s (KCl). In addition, we compared the amounts of Ca\(^{2+} \) that were released during both types of [Ca\(^{2+} \)]\(_s\), transients. For this purpose, we calculated the area under the curve as a measure of the total amount of Ca\(^{2+} \) released into the sarcoplasm. We first set the basal [Ca\(^{2+} \)]\(_s\), signal of each normalized recording to zero by subtracting the value 1 from each data point. Subsequently, individual [Ca\(^{2+} \)]\(_s\), transients were integrated to obtain numerical values (arbitrary units\(_s\)). In doing so, we found that the relative amount of Ca\(^{2+} \) released during KCl treatment was 100 times larger than during a single spontaneous transient (15.16 versus 0.152 arbitrary units\(_s\)). Occasionally, a biphasic increase in [Ca\(^{2+} \)]\(_s\), was observed upon application of KCl (wild-type, \( n = 3 \) of 12; and CK\(^{-/-} \), \( n = 6 \) of 14) (Fig. 4C). This was the result of a fast burst of small [Ca\(^{2+} \)]\(_s\), transients superimposed on the rising phase of the large [Ca\(^{2+} \)]\(_s\), signal.

**Role of CK in KCl-induced [Ca\(^{2+} \)]\(_s\), Transients—**To investigate the effects of CK deficiency on Ca\(^{2+} \) handling in KCl-induced [Ca\(^{2+} \)]\(_s\), transients, we compared Ca\(^{2+} \) removal kinetics between wild-type (\( n = 12 \)) and CK\(^{-/-} \) myotubes (\( n = 14 \)). Automated exponential ﬁtting was not possible for all experimental data sets due to the presence of noise. We therefore ﬁtted a straight line to the steepest part of the decline phase of the transients (Fig. 4A, Linear decay) and used the absolute slope of this ﬁt as an approximation of the (linear) Ca\(^{2+} \) removal rate. The maximal Indo-1 ratio reached (\( R_{\text{max}} \)) (Fig. 4A) was used as a measure of the amplitude of the transient. Plotting the linear calcium removal rate as a function of \( R_{\text{max}} \) revealed a positive correlation between these parameters in wild-type and CK\(^{-/-} \) myotubes (Fig. 4D). In both cell types, this correlation was linear (wild-type, Pearson's \( R = 0.91 \); and CK\(^{-/-} \), \( R = 0.93 \)). For both lines, the intercept differed not signiﬁcantly from zero. If [Ca\(^{2+} \)]\(_s\), does not rise (i.e. if there are no observable changes in [Ca\(^{2+} \)]\(_s\), and therefore \( R_{\text{max}} = 0 \), the linear Ca\(^{2+} \) removal rate in our experimental trace will also be zero. This means that by subtracting 1 from each \( R_{\text{max}} \) value to correct for the normalization (thereby setting baseline ratio signals to zero), both regression lines can be forced through the origin (0,0). We observed that, independent of amplitude, the removal rate was higher in wild-type myotubes than in CK\(^{-/-} \) myotubes (Fig. 4D, ● versus ○, respectively). This is reﬂected by an increased slope of the ﬁtted line in wild-type cells (wild-type, slope = \( 0.09 \pm 0.005 \) s\(^{-1} \); and CK\(^{-/-} \), slope = \( 0.065 \pm 0.002 \) s\(^{-1} \); \( p = 0.05 \)) (Fig. 4D, inset).

Therefore, wild-type myotubes remove Ca\(^{2+} \) faster than CK\(^{-/-} \) myotubes in KCl-induced [Ca\(^{2+} \)]\(_s\), transients. This, combined with the interpretation of Fig. 4 (A–C) described above, reveals that CK is important in the supply of ATP to plasma membrane Ca\(^{2+} \)-ATPases in KCl-induced Ca\(^{2+} \) transients.

**Electrical Stimulation of Myotubes—**Having noted the profound differences in Ca\(^{2+} \) handling during spontaneous and KCl-induced events, the effect of CK deficiency on Ca\(^{2+} \) handling was analyzed in more detail. To this end, we developed an experimental setup that allowed us to electrically induce [Ca\(^{2+} \)]\(_s\), rises in silent myotubes (i.e. temporarily not displaying spontaneous [Ca\(^{2+} \)]\(_s\), transients). To prevent cell damage, we first determined the minimal stimulation voltage required for the induction of [Ca\(^{2+} \)]\(_s\), transient rise. In doing so, we found that a 10-ms pulse of 16 V was optimal to induce a [Ca\(^{2+} \)]\(_s\), rise with kinetics resembling that of a spontaneous [Ca\(^{2+} \)]\(_s\), transient (Fig. 5A; see also Ref. 34). To mimic increasing workloads, we used pulse protocols with frequencies ranging from 0.1 to 10 Hz.

**Role of SR and SR Ca\(^{2+} \)-ATPase in Electrically Induced [Ca\(^{2+} \)]\(_s\), Transients—**As observed for spontaneous [Ca\(^{2+} \)]\(_s\), tran-
sients, application of thapsigargin (2 \(\mu\)M) significantly reduced the Ca\(^{2+}\) removal rate following a 2-s period of stimulation at 5 Hz in wild-type myotubes (duration of thapsigargin presence of 2 min) (Fig. 5B). This was reflected by an increase in the decay constant from 0.242 \(\pm\) 0.006 s (\(R^2 = 0.979\)) to 0.806 \(\pm\) 0.0003 s (\(R^2 = 0.973\)) and an elevated post-stimulatory basal Ca\(^{2+}\) level (experiment performed at room temperature).

In the absence of extracellular Ca\(^{2+}\), periodic electrical stimulation (at 5 Hz over 2 s) resulted in a progressive decrease in the amplitude of the induced [Ca\(^{2+}\)]\(_{i}\) transients (Fig. 5C, wild-type data shown). However, it took 3 min before the electrically mobilizable Ca\(^{2+}\) store was depleted. This finding is compatible with an efficient re-uptake of Ca\(^{2+}\) into the SR and a minimal loss of Ca\(^{2+}\) across the plasma membrane.

Ryanodine (10 \(\mu\)M) gradually abolished the electrically induced [Ca\(^{2+}\)]\(_{i}\) transients (Fig. 5D). In addition, this drug slowly increased the base-line [Ca\(^{2+}\)]. To assess the possible involvement of mitochondria in the generation of Ca\(^{2+}\) transients in the myotubes, we applied carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone (FCCP) to the cells. This protonophore dissipates the mitochondrial membrane potential and abolishes mitochondrial ATP synthesis. Fig. 5E shows that FCCP (50 \(n\)M) elevated the base-line [Ca\(^{2+}\)], and ultimately prevented electrical induction of [Ca\(^{2+}\)]\(_{i}\) transients within 1–2 min. Qualitatively, no differences concerning excitability, effects of thapsigargin, omission of extracellular Ca\(^{2+}\), ryanodine treatment, and FCCP application were observed between wild-type and CK\(^{-/-}\) myotubes (wild-type, \(n = 10\); and CK\(^{-/-}\), \(n = 13\)).

**Role of CK in Electrically Induced [Ca\(^{2+}\)]\(_{i}\), Transients—** We used the stimulation protocol shown in Fig. 5A to estimate Ca\(^{2+}\) removal rates in wild-type and CK\(^{-/-}\) myotubes as a function of workload. For each cell, the individual transients obtained at 0.1 Hz (wild-type, 19 myotubes; and CK\(^{-/-}\), 16 myotubes) were aligned on their maximal amplitude, after which the average ratio signal was calculated for each time point. Similarly, individual transients induced at 1 Hz were averaged (at least three transients/myotube) (wild-type, 20 myotubes; and CK\(^{-/-}\), 17 myotubes). Because at 5/10 Hz the transients were superimposed on an elevated basal [Ca\(^{2+}\)]\(_{i}\), only declines at the end of each burst were analyzed. The average traces obtained after 5- and 10-Hz stimulation were identical. Therefore, data were pooled for these stimulation frequencies. In CK\(^{-/-}\) myotubes (Fig. 6, A–C), this effect became more pronounced at higher stimulation frequencies and resembled that induced by thapsigargin treatment (Fig. 5B). The difference in removal rate was quantified by analysis of the
individual [Ca\(^{2+}\)]\(_s\) transients used for the construction of Fig. 6 (A–C). This analysis revealed decay constants of \(0.097 \pm 0.002\) s (\(R^2 = 0.998\)) and \(0.137 \pm 0.006\) s (\(R^2 = 0.989\)) for wild-type and CK\(^{-/-}\) myotubes, respectively, at 0.1 Hz (Fig. 6D). This increase in decay constant became more prominent at stimulation frequencies of 1 Hz (wild-type, 0.103 \(\pm\) 0.003 s and \(R^2 = 0.994\); and CK\(^{-/-}\), 0.160 \(\pm\) 0.005 s and \(R^2 = 0.995\)) and 5/10 Hz (wild-type, 0.111 \(\pm\) 0.008 s and \(R^2 = 0.996\); and CK\(^{-/-}\), 0.162 \(\pm\) 0.006 s and \(R^2 = 0.993\)).

To assess whether this reduced Ca\(^{2+}\) removal was associated with a reduced filling state of the SR, the area under each transient (arbitrary units/s) was calculated and used as an estimate of the amount of Ca\(^{2+}\) released (see above). To allow comparison, the average amount of Ca\(^{2+}\) released per transient at 0.1 Hz was set at 100%, a value to which all other values were related. Fig. 6E shows that in CK\(^{-/-}\) (but not wild-type) myotubes, gradually less Ca\(^{2+}\) was released with increasing stimulation frequency. The values presented were obtained from three wild-type and three CK\(^{-/-}\) myotubes that displayed stable base-line signals during the whole experiment. The number of [Ca\(^{2+}\)]\(_s\) transients included in the analysis was similar for wild-type (0.1 Hz, 9; 1 Hz, 80; and 5/10 Hz, 137) and CK\(^{-/-}\) (0.1 Hz, 9; 1 Hz, 95; and 5/10 Hz, 133) myotubes. Combination of the data presented in Fig. 6 (D and E) revealed a correlation between the amount of Ca\(^{2+}\) released per transient and the removal rate (Fig. 6F). The slower this rate, the less Ca\(^{2+}\) was released. The figure nicely demonstrates that CK\(^{-/-}\) myotubes, in contrast to wild-type myotubes expressing this
kinase, are unable to maintain an adequate removal rate at higher workloads (1 and 5/10 Hz). As a consequence, these myotubes release less Ca\(^{2+}\) per transient during high frequency stimulation.

**DISCUSSION**

In skeletal muscle, Ca\(^{2+}\) is an important physiological mediator of contraction, signal transduction, protein metabolism, differentiation, and growth (19). As far as contractile properties are concerned, speed of muscle contraction and relaxation critically depend on the spatial arrangement of components belonging to the Ca\(^{2+}\) handling apparatus. Moreover, Ca\(^{2+}\) directly stimulates mitochondrial ATP production in primary cultures of skeletal muscle myotubes (35), regulates myoblast fusion during myogenesis (36, 37), and is involved in the reprogramming of muscle gene expression via calcineurin (38). This means that the presence, functioning, and regulation of proteins involved in Ca\(^{2+}\) signaling and handling are of paramount importance in muscle development and physiology. During excitation-contraction coupling, considerable amounts of ATP are required for SERCA-mediated re-sequestration of Ca\(^{2+}\) into the SR (2). Because ATP is rapidly replenished by the CK/phosphocreatine circuit, we hypothesized earlier (10) that the CK system has a direct role in the regulation of both Ca\(^{2+}\) sequestration and release in skeletal muscle.

Conversely, altered Ca\(^{2+}\) kinetics could be a factor to explain the effect that ablation of both M-CK and ScCKmit genes has on physiological (force/fatigue) performance and on the metabolic and cytoarchitectural properties of muscles in our mutant CK mouse model(s). CK\(^{-/-}\) muscles undergo a general transition toward a “slow” phenotype, illustrated by an increase in mitochondrial volume in fast muscle fibers and “global” changes in transcriptome and proteome profiles of both fast and slow fibers (for more details on the phenotypic characterization of mutant CK mice, see Refs. 10, 14, and 15 and references therein). To better understand this putative role of Ca\(^{2+}\) in metabolic signaling cascades, we need to provide an adequate picture of the kinetics and subcellular distribution of Ca\(^{2+}\) transients, especially under non-strenuous conditions of muscle use as will occur under normal housing conditions of laboratory animals. In this study, we started to compare spontaneous and electrically induced [Ca\(^{2+}\)]\(_i\) signals in wild-type and CK\(^{-/-}\) myotubes cultured in vitro. Furthermore, we drew parallels to events that occur when KCl is used for full depolarization of muscle cells to assess whether CK deficiency alters Ca\(^{2+}\) sequestration and release. To visualize [Ca\(^{2+}\)]\(_i\), in time, Indo-1-loaded myotubes were subjected to high speed (30 Hz) confocal laser scanning microscopy. In both wild-type and CK\(^{-/-}\) preparations, spontaneous [Ca\(^{2+}\)]\(_i\) transients were observed that were associated with fast unidirectional [Ca\(^{2+}\)]\(_i\) waves, transecting the nucleoplasm. Because transients could be induced by KCl depolarization and displayed a higher frequency upon increasing temperature, the membrane potential of the myotube likely controls the temporal firing pattern. Because the membrane potential in myoblasts changes con-
comitantly with fusion, the temporal pattern of the [Ca\(^{2+}\)]
transients might be important for myogenesis (39). We showed earlier that the ultrastructural composition and mitochondrial volume are similar in wild-type and CK-deficient myotubes. This, combined with our current finding that the temporal Ca\(^{2+}\) patterns of CK-deficient and wild-type myotubes are similar, strongly suggests that the in vitro differentiation of myotubes is not influenced by CK absence.

The presence of spontaneous contractions in myotubes displaying Ca\(^{2+}\) transients, combined with the dependence on functional SERCAs, extracellular Ca\(^{2+}\), and ryanodine receptors, indicates that a functional excitation-contraction coupling mechanism is present in our myotube preparations (19). Given the homogeneous Ca\(^{2+}\) kinetics throughout the sarcoplasm in a single myotube, comparison of Ca\(^{2+}\) removal rates between individual myotubes was possible. Because this removal was monoeXponential, we conclude that the action of a single major removal process is responsible for the decline. The exponential decay constant \(\mu\), inversely proportional to the rate of Ca\(^{2+}\) removal, was used to quantify the Ca\(^{2+}\) removal rate. During spontaneous activity, Ca\(^{2+}\) removal was significantly slower in CK\(^{-}\) preparations. This shows that a functional CK system is essential for optimal Ca\(^{2+}\) removal during spontaneous activity.

Because the rate of Ca\(^{2+}\) removal was rapidly reduced by thapsigargin treatment and correlated with the amount of Ca\(^{2+}\) released from the SR during electrical stimulations, we considered it to be a quantitative readout for SERCA pump activity. This approach is supported by other studies presenting evidence that (i) the SERCA pump takes primacy over Na\(^+\)/Ca\(^{2+}\) exchange and plasma membrane Ca\(^{2+}\)-ATPase action at the plasma membrane in both cardiac and skeletal muscle (40, 41), (ii) SERCA pumping rate directly depends on ATP supply (19), and (iii) CK is of paramount importance in mediating this supply (6). The SR has a large Ca\(^{2+}\) storage capacity, which is effectively maintained by SERCA pumps because neither thapsigargin treatment nor omission of extracellular Ca\(^{2+}\) acutely abolished spontaneous or electrically induced [Ca\(^{2+}\)], transients.

KCl depolarization induced large [Ca\(^{2+}\)]
transients. This Ca\(^{2+}\) originated from the SR because the amplitude and duration of the transients were independent of extracellular Ca\(^{2+}\). In KCl-induced [Ca\(^{2+}\)], transients, Ca\(^{2+}\) was mainly extruded across the plasma membrane because a second KCl challenge under Ca\(^{2+}\)-free conditions induced a 75% smaller transient, featuring a lower Ca\(^{2+}\) release rate. These findings reveal that the filling state of the SR is reduced during KCl treatment, in agreement with other studies showing that the amount of Ca\(^{2+}\) released from the SR by a stimulus directly depends (probably in a highly nonlinear fashion) (42) on its filling state (43). The amount of Ca\(^{2+}\) released in a single spontaneous transient was ~100 times smaller than during KCl treatment. Therefore, the Ca\(^{2+}\) storage capacity of the SR, combined with effective SERCA-mediated refill, is sufficient to allow sustained spontaneous activity. Also during KCl treatment, Ca\(^{2+}\) was removed more slowly in CK\(^{-}\) myotubes. Because this removal primarily occurred across the plasma membrane, this points to an impaired plasma membrane Ca\(^{2+}\)-ATPase function.

To allow study of the effects of CK deficiency on Ca\(^{2+}\) signaling at increasing workloads, a setup for active electrical depolarization was developed. Each electrical stimulation induced a single rapid [Ca\(^{2+}\)] transient that displayed kinetics similar to those of spontaneous transients. Results obtained with thapsigargin, Ca\(^{2+}\)-free medium, and ryanodine were similar to those in spontaneously active myotubes, again demonstrating the presence of a functional excitation-contraction coupl

plcing and predominance of SERCA-mediated SR refilling. By increasing the stimulation frequency, cells were subjected to higher Ca\(^{2+}\) loads. In wild-type myotubes, neither the Ca\(^{2+}\) removal rate nor the amount of Ca\(^{2+}\) released per transient changed as a function of stimulus intensity. This shows that ATP demand and supply are well balanced also at high Ca\(^{2+}\) loads. In contrast, CK\(^{-}\) myotubes always had a lower Ca\(^{2+}\) removal rate than wild-type myotubes. Strikingly, in CK\(^{-}\) myotubes, both Ca\(^{2+}\) removal rate and amount of Ca\(^{2+}\) released decreased progressively with higher stimulus intensity. This correlation strengthens the validity of our analysis method and shows that the SR filling state is reduced in CK\(^{-}\) myotubes at higher stimulation frequencies. This reduced filling could have direct consequences for myotube maturation (which is associated with the occurrence of spontaneous Ca\(^{2+}\) transients) (44) given the hypothesized role of Ca\(^{2+}\) as an intra-SR messenger (45). The observation that Ca\(^{2+}\) removal becomes progressively impaired in CK\(^{-}\) myotubes as a function of stimulus intensity shows that the CK system is especially important under conditions of high ATP demand. In the latter case, the cytosolic phosphorylation potential can be buffered at a higher level in wild-type myotubes than in cells devoid of the CK system.

Because mitochondrial ATP generation accounts for 60% of the energy production in differentiated myotubes (46), the observed metabolic and cytoarchitectural remodeling in CK-deficient muscle might serve as an effort to counterbalance the CK defect (10, 11, 14, 15). Our FCCP results show that functional mitochondria are especially important during excitation-contraction coupling; and in this respect, the remodeling likely functions as a means to reduce the distance between sites of ATP production (mitochondria) and ATP consumption (e.g., SERCAs). To prove this hypothesis and to provide further evidence for a functional connection among energy transfer, Ca\(^{2+}\) signaling, and the adaptational changes in our mutant CK muscles, we need also more insight in the cellular compartmentalization of ATP and ADP and the local ATP/ADP ratio in the direct vicinity of the SERCA pumps. Of importance in this respect is the proposed existence of functional complexes of mitochondria with Ca\(^{2+}\) ATPases and SR in muscle cells (25).

Acknowledgments—We thank F. Oerlemans for technical assistance and Dr. J. E. Morgan (Department of Muscle Cell Biology, Imperial College School of Medicine, Hammersmith Hospital, London) for advice on muscle cell culture.

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