

Creatine supplementation upregulates excitation-contraction coupling in C2C12 myotubes

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Abstract: The main goal of this work was to investigate whether creatine (Cr) might be able to regulate skeletal muscle excitation-contraction (EC) coupling. Myotubes from a C2C12 cell line were exposed to Cr (25 mM, 2–4 hours). Subsequently, the activity of L-type Ca^{2+} channels and voltage-gated Ca^{2+} release (VGCR) were investigated using the whole-cell patch-clamp technique. Cr upregulated VGCR by 2.4-fold, in the absence of major alterations in L-type Ca^{2+} channel activity, the extent of caffeine-induced sarcoplasmic reticulum Ca^{2+} release, or the termination kinetics of Ca^{2+} transients. Thus, stimulation of VGCR cannot be explained by upregulation of the activity of either L-type Ca^{2+} channels or sarcoplasmic reticulum Ca^{2+} -ATPase. We also investigated possible long-term regulation of L-type Ca^{2+} channels by Cr. However, chronic treatment with Cr (2–4 days) affected neither the density of I_{CaL} nor the corresponding voltage-dependence of activation. The later result was obtained in the face of a 1.7-fold stimulation of myogenesis. Therefore, it cannot be explained by possible desensitization of Cr metabolism. These data could suggest that the functional expression of L-type Ca^{2+} channels is not affected by prior acute stimulation of VGCR. Previous work has shown that Cr supplementation enhances muscle strength. Thus, it will be interesting to investigate whether this effect can be at least partially explained by acute stimulation of VGCR.

Keywords: L-type calcium channel, ryanodine receptor, intracellular calcium

Introduction

In skeletal muscle, excitation-contraction (EC) coupling depends on a physical interaction between two classes of calcium channels, ie, dihydropyridine receptors (DHPRs, also known as L-type Ca^{2+} channels), located at the sarcolemma; and ryanodine receptors (RyR1s), located at the sarcoplasmic reticulum (SR). This process involves three principal events, ie, electrical depolarization of the sarcolemma, intramembrane charge movement of the DHPR voltage sensor, and SR Ca^{2+} release via activated RyR1s (termed voltage-gated Ca^{2+} release or VGCR). Subsequently, a transient increase in myoplasmic Ca^{2+} concentration stimulates the contractile machinery and muscle contraction.^{1,2}

Creatine (Cr) is an amino acid that can be obtained from food and by *de novo* synthesis. It is converted to phosphocreatine (PCr) by the enzyme creatine kinase (CK), using ATP from oxidative phosphorylation as a substrate. High rates of ATP hydrolysis promote the reverse chemical reaction (ie, ATP synthesis from ADP and PCr). Thus the Cr-CK-PCr system supplies ATP for a number of processes requiring high levels of energy consumption.³ Indeed it has been reported that oral Cr supplementation stimulates contractile force in humans, eg, in high-intensity exercise, in the elderly, and in patients with muscle dystrophy and Parkinsonism.^{4–7}

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Effects that have been reported for the Cr-CK-PCR system in skeletal muscle cells include enhancement of the activity of SR Ca^{2+} ATPase (SERCA),^{8,9} stimulation of contractile machinery,¹⁰ promotion of muscle development or myogenesis,^{11,12} and stabilization of physiologic levels of myoplasmic Ca^{2+} concentration.¹³ However, there have been no studies to date that have measured the potential effects on EC coupling, even though this process is critical to force development under both physiologic and pathologic conditions. Therefore we have investigated the potential effects of Cr supplementation on EC coupling, using myotubes from a C2C12 cell line. Interestingly, short-term incubation with Cr potentiated VGCR, in the absence of an increased SR Ca^{2+} load, higher L-type Ca^{2+} channel activity, or SERCA stimulation. As explained in the discussion, these observations point to a relatively unexpected overactivity of RyR1 during EC coupling.

Methods

Cell culture

Myotubes were obtained from a C2C12 cell line (ATCC number: CRL-1772). Briefly, C2C12 myoblasts were kept under proliferation conditions and subcultured every other day. The proliferation medium consisted of DMEM solution supplemented with fetal bovine serum (20%), penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and L-glutamine (4 mM).

To generate myotubes, ~ 3000 myoblasts/ cm^2 were plated on 35 mm Petri dishes. Twenty-four hours later (day 0), the proliferation medium was replaced by differentiation medium, which was similar to proliferation medium, but contained 2%

horse serum instead of 20% fetal bovine serum. Three days thereafter, the culture medium was changed to either standard differentiation medium (control cells) or differentiation medium supplemented with 25 mM of Cr (treated cells). Exposure to Cr lasted either 2–4 hours (short-term treatment) or 2–4 days (long-term treatment). All cells were kept at 37°C in saturated air humidity and 5% CO_2 .

Voltage-clamp experiments

The whole-cell patch-clamp technique was used to investigate the activity of L-type Ca^{2+} channels, as described elsewhere.^{14,15} Briefly, myotubes were transferred from a CO_2 incubator to a recording chamber containing 2 mL of extracellular recording solution (see below). Patch-clamp electrodes were filled with ~ 10 μL of internal recording solution, and exhibited electrical resistances of ~ 1.5 $\text{M}\Omega$. The series resistance was electronically compensated (up to $\sim 85\%$), and the cell membrane capacitance (C_m) was estimated using the method described by Meza and Cols.¹⁶

The holding potential (HP) was -80 mV. Except for results shown in Figure 3, a 1 sec prepulse to $+20$ mV preceded application of test pulses. The prepulse was designed to eliminate a remanent Na^+ current, as well as possible contamination by T-type Ca^{2+} current.¹⁷ The duration of test pulses was 30 msec for simultaneous measurements of both I_{CaL} and intracellular Ca^{2+} transients (Figures 1 and 2). In contrast, test pulses were used to investigate only I_{CaL} lasted 200 msec (Figure 5). The corresponding sampling frequencies were 10 kHz (30 msec pulses) and 5 kHz (200 msec pulses). I_{CaL} was analogically filtered, using a 4-pole low-pass Bessel filter (at 2 kHz); normalized by C_m , plotted as a function of mem-

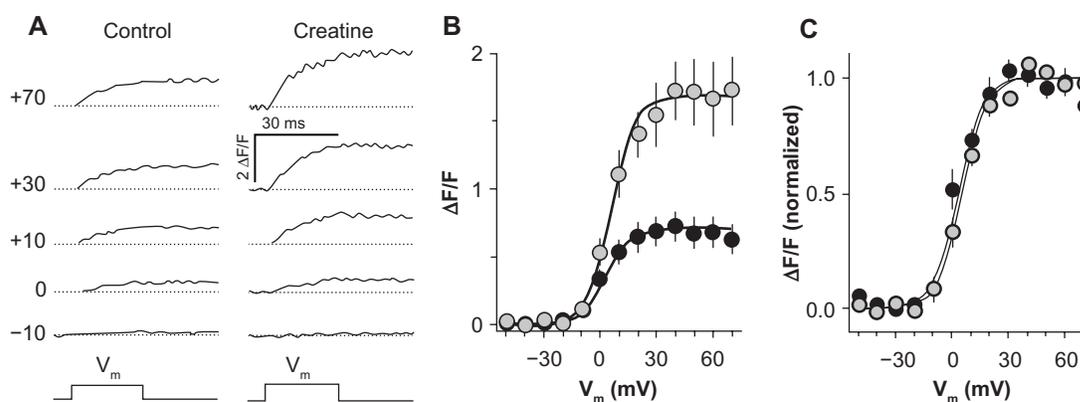


Figure 1 Voltage-gated SR Ca^{2+} release. **A)** Representative traces of intracellular Ca^{2+} transients elicited in control and creatine-treated myotubes. The values of test membrane potential (V_m) are indicated at the left. **B)** Average amplitude of Ca^{2+} transients elicited as in A. The amplitude of the transients was measured at the end of test pulses, averaged, and plotted as a function of V_m . Continuous lines represent theoretical functions, estimated according to Equation 2 and the Boltzmann parameters given in Table 1. **C)** Normalized values of Ca^{2+} transients. The absolute values of Ca^{2+} transients from each myotube were normalized with respect to their corresponding maximal value (ie, $(\Delta F/F)_{\text{max}}$). The continuous lines from B were redrawn, following normalization by the corresponding $(\Delta F/F)_{\text{max}}$ values given in Table 1. Treatment with creatine lasted 2–4 hours.

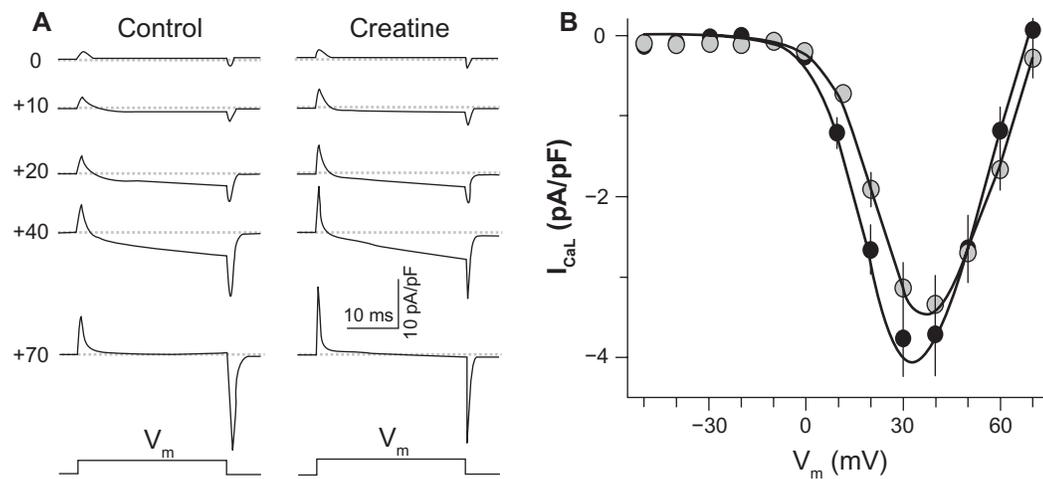


Figure 2 L-type Ca²⁺ currents. **A)** Examples of Ca²⁺ currents elicited in control and creatine-treated myotubes. The values of test membrane potential (V_m) are given at the left. **B)** Average current-to-voltage relationships for Ca²⁺ currents elicited as in A. The peak amplitude of Ca²⁺ currents was averaged and plotted as a function of V_m. Continuous lines were created using the Equation 1 and parameters given in Table 2. The average values of C_m were almost identical in control and treated myotubes (65 ± 5 pF and 72 ± 5 pF, respectively). The same pool of cells is used here as in Figure 1.

brane potential (V_m), and fitted according to the following Boltzmann equation:

$$I = G_{\max} (V_m - V_{\text{rev}}) / \{1 + \exp [(V_{G1/2} - V_m) / k_G]\} \quad (1)$$

G_{max} represents the maximal conductance, V_{rev} is the apparent reversal potential, V_{G1/2} is the potential required to activate 50% of G_{max}, and k_G is a slope factor.

Simultaneous measurements of I_{CaL} and voltage-gated Ca²⁺ transients were performed using microfluorometry of Fluo-4, as described elsewhere.¹⁵ Briefly, the internal recording solution was supplemented with K₅Fluo-4 (0.2 mM). The dye was excited using a 100 W arc mercury lamp, an excitation filter of 470–490 nm, and a dichroic mirror centered at 505 nm. The emitted fluorescence was acquired using an emission filter (515–550 nm) and a photomultiplier tube (Hamamatsu, R1527P). The sampling frequency was 10 kHz. Fluorescence was normalized with respect to its corresponding basal value, which was determined just before membrane depolarization (ΔF/F). Subsequently, the estimated (ΔF/F) values were plotted as a function of V_m, and fitted according to the following Boltzmann equation:

$$\Delta F/F = (\Delta F/F)_{\max} / \{1 + \exp(V_{F1/2} - V_m) / k_F\} \quad (2)$$

where (ΔF/F)_{max} represents the maximum amplitude of ΔF/F, V_{F1/2} is the potential required to obtain 0.5 of (ΔF/F)_{max}, and k_F is a slope factor.

In Figure 3, caffeine (30 mM) application was preceded by a test pulse of 30 msec to +70 mV. For these experiments, the sampling frequency of the fluorescence signal was 2 kHz.

Caffeine was locally applied using a fast perfusion system (Warner Instruments, LLC, Hamden, CT). The delay between the electronic command for caffeine application and SR Ca²⁺ release was ~5–10 sec.

Immunofluorescence

Myotubes chronically exposed to Cr (2–4 days) and the corresponding controls were fixed with 4% paraformaldehyde (15 minutes, at 0°C), washed 3 times with phosphate-buffered saline, and kept for 1 hour in blocking buffer (consisting of phosphate-buffered saline, 0.3% triton, and 5.5% normal goat serum). The fixed myotubes were incubated overnight in the presence of anti-phospholamban antibody (Affinity Bioreagents, dilution 1:1000). Primary antibody was detected with Alexa 488-conjugated anti-rabbit (Invitrogen, dilution 1:1000). The nuclei were stained using Hoechst (1 μM, 15 minutes). Finally, the samples were mounted onto a slide using vectashield.

Images were taken using a digital camera (Canon PowerShot, A720 IS), which was mounted onto an inverted microscope (Olympus, IX71). The excitation filter used to detect secondary antibody consisted of an excitation filter (460–500 nm), a dichroic mirror (505 nm), and an emission filter (510–550 nm). Hoechst was excited at 360–370 nm, using an excitation filter and dichroic mirror centered at 380 nm.

For each field, fluorescence images corresponding to Hoechst and anti-phospholamban antibody were stored on the hard drive of a PC, and subsequently processed with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). Basically, the matching images were

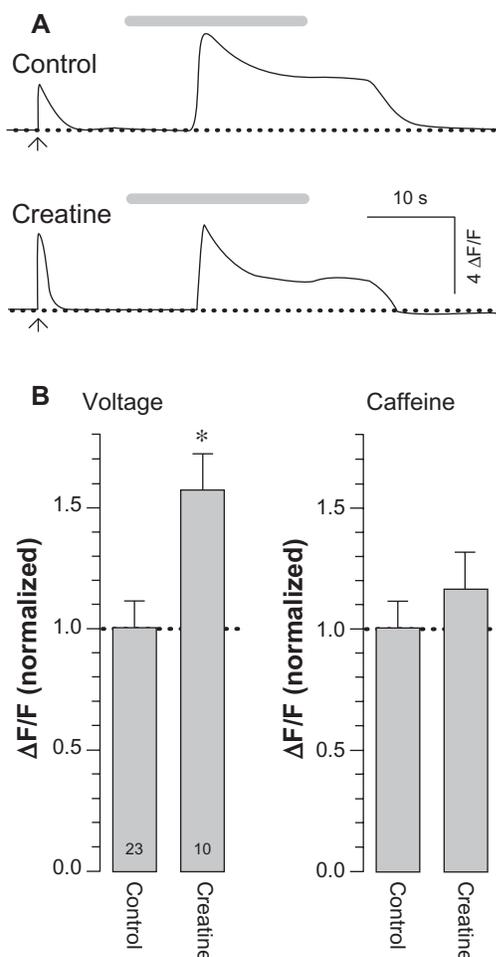


Figure 3 Voltage- and caffeine-induced SR Ca²⁺ release. **A**) Representative Ca²⁺ transients induced by voltage and caffeine, in both control and creatine-treated myotubes. The gray bars represent 20 sec of caffeine applications, which were preceded by 30 msec of membrane depolarization (ie, from -80 mV to $+70$ mV, arrows). **B**) Normalized amplitude of Ca²⁺ transients elicited as in A. Absolute values of Ca²⁺ transients were normalized by the corresponding mean values obtained from control myotubes (ie, 2.2 ± 0.3 ΔF/F for voltage, and 3.5 ± 0.5 ΔF/F for caffeine). The numbers of investigated myotubes are given in B. Treatment with creatine lasted 2–4 hours.

overlapped, and the resulting ensemble used to calculate the fusion index (defined as the percentage of nuclei in myotubes with respect to the total number of nuclei).

Recording solutions

To record only I_{CaL} , the following internal solution was used (in mM): 145 Cs aspartate, 10 CsCl, 10 Cs₂-EGTA, and 10 HEPES. In contrast, the internal recording solution for simultaneous measurements of Ca²⁺ transients and I_{CaL} , consisted of (in mM): 145 Cs-aspartate, 10 CsCl, 0.1 Cs₂-EGTA, 5 Mg-ATP, 0.2 K₅Fluo-4, and 10 HEPES. All experiments were performed in the presence of the following external solution (in mM): 150 tetraethylammonium chloride, 10 CaCl₂, and 10 HEPES. The pH was adjusted to 7.3 in all recording solutions.

Data represent the median \pm SEM. Statistical significance was determined by unpaired Student's t-test ($P < 0.05$). All experiments were performed at room temperature (22 – 24°C).

Results

Short-term creatine supplementation upregulates voltage-gated SR Ca²⁺ release

VGCR represents a critical step in EC coupling and force development.^{1,2} Therefore, we decided to investigate whether a short-term (2- to 4-hour) supplementation of culture medium with Cr might be able to regulate VGCR. Specifically, we simultaneously recorded I_{CaL} and intracellular Ca²⁺ transients, in both control and Cr-treated myotubes. Figure 1A shows representative traces of Ca²⁺ transients recorded from a control and a treated myotube, where the amplitude of the transients is higher in the treated myotube. On average, the maximal amplitude of Ca²⁺ transients, or $(\Delta F/F)_{max}$, was $\sim 140\%$ higher in treated myotubes (Figure 1B and Table 1). Interestingly, this effect is not due to potential alterations in the voltage-dependence of the SR Ca²⁺ release process, given that Cr did not affect the Boltzmann parameters describing activation curves of the transients (ie, k and $V_{F1/2}$, see Figure 1C and Table 1).

Short-term myotube exposure to creatine does not modulate L-type Ca²⁺ channels

L-type Ca²⁺ channels are important for EC coupling, because they represent voltage sensors that activate SR Ca²⁺ release.^{18,19} Therefore, in myotubes from Figure 1 we also analyzed the activity of these channels. Figure 2A shows examples of I_{CaL} that were obtained from myotubes cultured as in Figure 1. The currents were normalized by cell membrane capacitance or C_m , to prevent potential alterations in cell size. In contrast with the drastic increase in the amplitude of the transients (Figure 1), the density of I_{CaL} was similar in control and treated myotubes. In fact, Cr did not produce major alterations in either the average current-to-voltage relationships (I – V curves, Figure 2B) or the correspond-

Table 1 Parameters of fitted $(\Delta F/F)$ - V curves (short-term treatment)

	$(\Delta F/F)_{max}$	$V_{F1/2}$ (mV)	k_F (mV)
Control (n = 18)	0.7 ± 0.1	6.4 ± 0.7	3.1 ± 1.9
Creatine (n = 12)	$1.7 \pm 0.2^*$	6.4 ± 0.4	5.6 ± 1.6

Notes: Parameters were obtained by fitting data in Figure 1B according to Boltzmann equation 2. The asterisk indicates a significant difference.

ing Boltzmann parameters (Table 2). Thus, Cr drastically stimulates VGCR, without significantly altering the activity of L-type Ca^{2+} channels.

Stimulation of VGCR is not due to increased SR Ca^{2+} content

In isolated SR vesicles, the Cr-CK-PCr system accelerates Ca^{2+} reuptake.^{8,9} Therefore, we speculated that the stimulus in VGCR (Figure 1) might be due to a possible higher SR Ca^{2+} content. However, results shown in Figure 3 strongly argue against this interpretation. Figure 3A shows representative Ca^{2+} transients induced by voltage (arrows) and caffeine (gray bars), in control and treated myotubes. As expected, in the treated myotube the amplitude of the voltage-gated Ca^{2+} transients (arrows) was higher compared with the control. In contrast, the amplitude of caffeine-induced Ca^{2+} transients (gray bars) was similar in both myotubes. On average, Cr significantly increased the amplitude of voltage-gated Ca^{2+} transients by up to ~60% of the control value (Figure 3 B). However, unexpectedly, this effect was in the absence of significant alterations in the amplitude of caffeine-induced Ca^{2+} release. The caffeine-sensitive SR Ca^{2+} releasable pool is generally used as an estimation of the load of SR Ca^{2+} . Therefore, these data suggest that the stimulus of Cr on VGCR cannot be explained by a potential increase in SR Ca^{2+} content.

Creatine does not alter termination kinetics of Ca^{2+} transients

The SR Ca^{2+} content was unaltered by Cr (Figure 3). This suggests pretreatment with this amino acid did not permanently affect the activity of SERCA. If this interpretation is correct, then Cr should not produce major alterations in the termination kinetics of Ca^{2+} transients, since this process strongly reflects the activity of SERCA.²⁰

Thus, we decided to investigate the duration of voltage-gated Ca^{2+} transients, as well as the corresponding decay rate. As can be observed in Figure 4A, Cr did not significantly alter these parameters. Thus, we can only conclude that

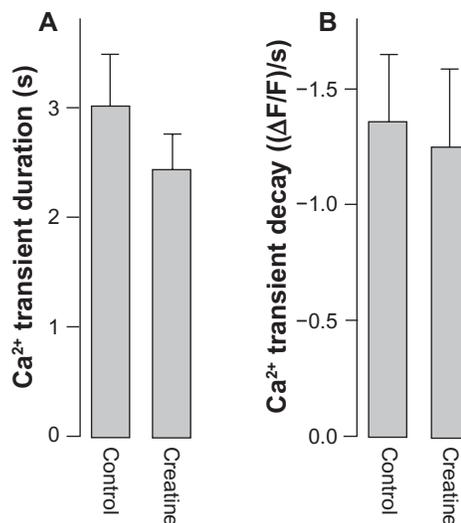


Figure 4 Termination kinetics of voltage-gated Ca^{2+} transients. Duration **A**) and decay rate **B**) of Ca^{2+} transients recorded as in Figure 3 (voltage). The duration was defined as the time interval between onset of depolarization and the time at which the signal decayed to 50% of peak amplitude, whereas decay rate represents the peak value of the negative derivative of the transient.

Cr did not accelerate Ca^{2+} transient decline, and therefore potentiation of VGCR cannot be explained by permanent stimulation of SERCA.

Functional expression of L-type Ca^{2+} channels is unaffected by chronic Cr supplementation

In primary cultured mice myotubes, Ca^{2+} release through RyR1 stimulates L-type Ca^{2+} channel expression.²¹ Therefore, we decided to investigate whether the short-term stimulation of VGCR might be able to upregulate the functional expression of L-type Ca^{2+} channels. Specifically, myotubes were allowed to differentiate in control conditions for four days, and were then grown for an additional 2–4 days in the presence of either standard differentiation medium (control) or differentiation medium supplemented with Cr. Subsequently, the activity of L-type Ca^{2+} channels was investigated. For these experiments, we used longer test pulses (ie, 200 msec, as opposed to 30 msec in Figures 1 and 2). Figure 5A shows examples of Ca^{2+} currents recorded with this protocol stimulation.

As can be seen in Figure 5B, the average density of I_{CaL} was similar in control and treated myotubes at all membrane potentials. Moreover, the Boltzmann parameters describing the voltage-dependence of L-type Ca^{2+} channels were practically identical in both experimental conditions (Table 3). Thus, we conclude that long-term Cr supplementation does not significantly affect the functional expression of L-type Ca^{2+} channels in C2C12 myotubes. The absence of effect was

Table 2 Parameters of fitted I–V curves (short-term treatment)

	G_{max} (nS/nF)	k_G (mV)	$V_{G1/2}$ (mV)	V_{rev} (mV)
Control (n = 18)	140 ± 19	7.3 ± 0.3	22.8 ± 1.0	69.2 ± 1.6
Creatine (n = 12)	125 ± 9	7.9 ± 0.3	27.4 ± 0.9*	72.8 ± 1.7

Notes: Parameters were obtained from fitting data in Figure 2B to Equation 1, and represent the mean ± SEM from the number of indicated experiments (n).

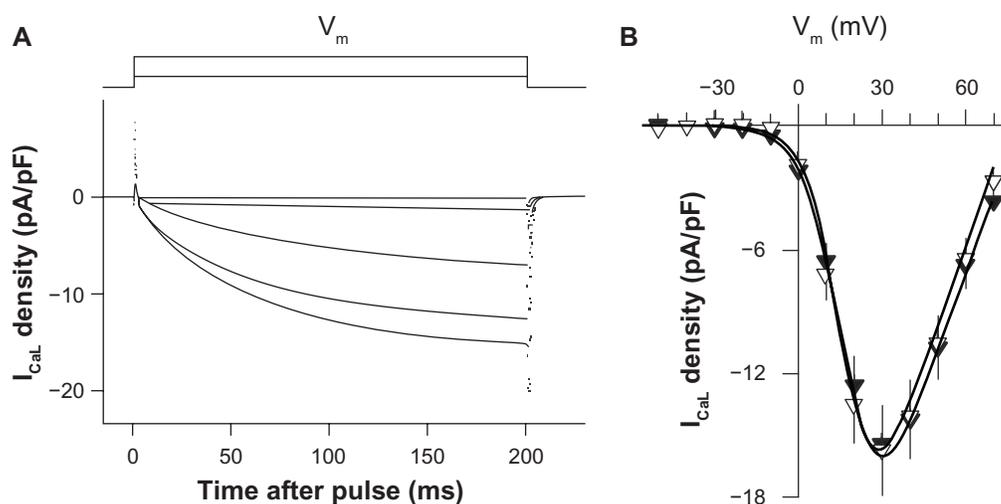


Figure 5 L-type Ca^{2+} currents in controls and chronically-treated myotubes. **A**) Representative family of I_{CaL} obtained from a control myotube. **B**) Average current-to-voltage relationships obtained from current traces recorded as in A. Symbols represent the average from a total of 17 control (opened triangles) and 19 creatine-treated (2–4 days, closed triangles) myotubes. Experimental data were fitted according to Boltzmann equation 1 and the resulting parameters are given in Table 3. The average values of C_m for control and treated myotubes were: 66 ± 12 pF and 71 ± 14 pF, respectively ($P = 0.8$).

not due to a possible desensitization of the Cr-CK-PCr system, because in parallel experiments Cr drastically increased myogenesis. Figure 6A shows representative microphotographs of control and Cr-treated (4 day) myotubes. Clearly, the treated myotubes are larger than controls. To quantify this morphologic effect we decided to estimate the fusion index. Basically, we labeled myotubes with anti-phospholamban antibody, and stained the nuclei with Hoechst (Figure 6B, green and blue signals, respectively). As a result, we found that Cr increased the fusion index by ~ 1.7 -fold. These data corroborate the observation that Cr stimulates myogenesis, and validate the negative results shown in Figure 5.

Discussion

This work shows that long-term treatment with Cr promotes myogenesis, without significantly altering the functional expression of L-type Ca^{2+} channels. Our data also indicate that short-term incubation with Cr stimulates VGCR. The later effect occurs in the absence of significant alterations in voltage dependence of the SR Ca^{2+} release process, cell size, L-type Ca^{2+} channel activity, caffeine-induced Ca^{2+} release, or termination kinetics of Ca^{2+} transients. As explained below, the results obtained from the short-term incubation

Table 3 Parameters of fitted I–V curves (long-term treatment)

	G_{max} (nS/nF)	k_G (mV)	$V_{G1/2}$ (mV)	V_{rev} (mV)
Control (n = 17)	386 ± 41	6.1 ± 0.3	16.8 ± 1.0	75.3 ± 2.6
Creatine (n = 19)	386 ± 39	6.9 ± 0.3	17.7 ± 1.1	78.5 ± 2.3

Notes: The results indicate Boltzmann parameters, obtained from fitting data on Figure 5B to equation 1.

can be interpreted to suggest that Cr upregulates RyR1 activity.

EC coupling depends on L-type Ca^{2+} channels, the voltage sensor activity of which results in SR Ca^{2+} release via RyR1.^{1,2,18,19} On the other hand, the releasable pool of SR Ca^{2+} strongly depends on Ca^{2+} reuptake by SERCA.²² Thus, a possible increase in the activity of voltage sensor, RyR1, or SERCA, might have contributed to the stimulation of VGCR by Cr. To speculate further with regard to the possible underlying molecular mechanism(s), it is important to clarify that Cr exerted its effect relatively quickly (in two hours). Thus, alterations in protein expression seem unlikely. Accordingly, the maximal conductance of L-type Ca^{2+} channels (G_{max}), which depends on the density of channels at the plasma membrane, was unaltered (Table 2). Moreover, Cr did not produce major alterations in the voltage dependency of activation of L-type Ca^{2+} channels (Table 2) or VGCR (Table 1). These observations strongly suggest that the stimulation of VGCR cannot be explained by a potential modulation of voltage sensor expression or function.

In skinned muscle fibers, PCr increases the amplitude of caffeine-induced Ca^{2+} transients, and accelerates the corresponding decay rate.²³ Moreover, in isolated SR vesicles, the Cr-CK-PCr system stimulates the activity of SERCA.^{8,9} Thus, in this work we hypothesized that increased Ca^{2+} reuptake and enhancement of the load of SR Ca^{2+} could explain the stimulation of VGCR by Cr. However, results shown in Figures 3 and 4 argue against this interpretation. This is because Cr altered neither the caffeine-sensitive

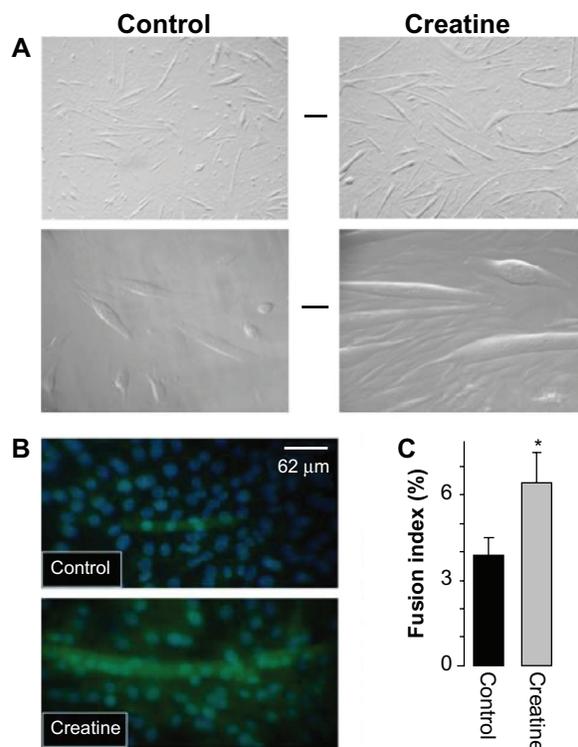


Figure 6 Chronic effects in myogenesis. **A)** Relief contrast microphotographs of control (left) and creatine-treated (4 day) myotubes. The calibration bars correspond to 1 mm (upper panels) and 62 μm (lower panels). **B)** Immunofluorescence images obtained from control and creatine-treated (two day) myotubes. The green signal corresponds to phospholamban antibody and the blue signal to the nuclei (see methods for further details). **C)** Fusion index estimated from images obtained as in B. Data represent the average from a total of 25 controls and 18 creatine-treated fields.

Ca²⁺ releasable pool, the duration of Ca²⁺ transients, nor the corresponding decay rate.

Alternatively, the stimulation of VGCR could be explained by a possible upregulation of RyR1 activity. ATP is a potent agonist of RyR1,^{24,25} and the Cr-CK-PCr system could have increased the availability of ATP in RyR1-enriched microdomains (ie, the junctional SR membrane). Unfortunately, the precise ATP binding site in RyR has not been identified,²⁶ which complicates the investigation of this hypothesis. In any event, ATP could have also stimulated RyR1 indirectly. For example, ATP could have promoted phosphorylation of inositides, which in turn bind to and stimulate RyR1.²⁷ Mg²⁺ represents another intriguing candidate which may mediate modulation of RyR1. This ion drastically inhibits RyR1 activity,^{28,29} and Cr supplementation could have decreased the concentration of free Mg²⁺ in microdomains nearby to RyR1 (due to promoting formation of the Mg-ATP quelate). Further work is obviously needed to investigate all these speculations.

It has been reported that normal intracellular PCr levels are almost entirely depleted during skeletal muscle fatigue.³⁰ This can be interpreted to suggest that Cr supplementation stimulates muscle performance by preventing and/or attenuating PCr depletion.³ Accordingly, contractile force stimulation by Cr has been observed not only under pathologic conditions,⁵⁻⁷ but also in high-intensity exercise.⁴ In intact (ie, non-patch-clamped) primary cultured myotubes, the basal intracellular PCr levels are approximately 50 mmol/mg protein, and can be increased up to ~2.5-fold following extracellular Cr supplementation.¹² Interestingly, in our patch-clamp experiments the cytosol was severely dialyzed, mimicking the PCr depletion observed during fatigue. Therefore it is possible that the remanent concentration of intracellular PCr could have been ~2.5-fold higher in Cr-treated myotubes compared with controls. Thus, even though the data derived from our studies may not necessarily be able to be extrapolated to adult human muscle, a relationship does exist in the sense that attenuation of PCr depletion apparently represents a shared step in the stimulation of both EC coupling and muscle performance.

On the other hand, it is currently unclear why in our experiments Cr did not regulate SERCA. The Cr-CK-PCr system stimulates SERCA only if the pump is working with low efficiency, and if the basal PCr levels are depleted.⁹ These observations partially explain the absence of effect on SR Ca²⁺ content, since in culture PCr was probably not depleted. Nevertheless, we expected an effect because PCr depletion most likely occurred during patch-clamp experiments. Further work is needed to solve this apparent contradiction.

The expression levels of L-type Ca²⁺ channels are decreased in myotubes from RyR1 knock-out (dyspedic) mice with respect to controls. Interestingly, the decreased levels can be restored following intranuclear microinjection of cDNA encoding RyR1. Conversely, they cannot be restored by transfecting an RyR1 mutant that releases Ca²⁺ poorly (~10% of normal). This suggests that Ca²⁺ release through RyR1 contributes to sustaining the expression levels of DHPR.²¹ It seemed reasonable, therefore, to speculate that short-term stimulation of VGCR by Cr could have exerted long-term upregulation of L-type Ca²⁺ channels. However, we found no evidence in support of this speculation (Figure 5). This apparent paradox could be explained if in culture Cr-treated myotubes can not release more Ca²⁺ than controls. Alternatively, Ca²⁺ release through RyR1s may stimulate expression levels of L-type Ca²⁺ channels only in primary culture, but not in clonal C2C12 myotubes. They are different

experimental models, and thus may not share identical signaling pathways. The fact that these two systems are different is emphasized by the observation that primary, but not C2C12 myotubes, express functional T-type Ca^{2+} channels (unpublished data).^{31,32} Thus, it will be interesting to investigate whether in primary cultured myotubes, Cr stimulates both VGCR and L-type Ca^{2+} channel expression.

In summary, our results indicate that Cr supplementation stimulates EC coupling in C2C12 myotubes. Enhanced SR Ca^{2+} release, via stimulated activity of RyR1, is the apparent underlying mechanism. These observations enhance our knowledge of the effects of the Cr-KC-PCr system in skeletal muscle cells. In the future, it will be interesting to investigate whether stimulation of EC coupling actually contributes to increased contractile force in individuals consuming Cr.

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Disclosures

The authors report no conflict of interest in this work.

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