Effect of creatine on contractile force and sensitivity in mechanically-skinned single fibers from rat skeletal muscle

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Running head: Creatine and contractile apparatus in single fibers
Abstract:

Increasing the intramuscular stores of total creatine (TCr = creatine (Cr) + Cr phosphate (CrP)) can result in improved muscle performance during certain types of exercise in humans. Initial uptake of Cr is accompanied by an increase in cellular water to maintain osmotic balance, resulting in a decrease in myoplasmic ionic strength. Mechanically-skinned single fibers from rat soleus (SOL) and extensor digitorum longus (EDL) muscles were used to examine the direct effects on the contractile apparatus of increasing [Cr], increasing [Cr] plus decreasing ionic strength, and increasing [Cr] and [CrP] with no change in ionic strength. Increasing [Cr] from 19 to 32 mM, accompanied by appropriate increases in water to maintain osmolality, had appreciable beneficial effects on contractile apparatus performance. Compared with control conditions both SOL and EDL fibers showed increases in Ca\textsuperscript{2+}-sensitivity (+0.061 ± 0.004 and +0.049 ± 0.009 pCa units, respectively) and maximum Ca\textsuperscript{2+}-activated force (to 104 ± 1 and 105 ± 1 %, respectively). In contrast, increasing [Cr] alone had a small inhibitory effect. When both [Cr] and [CrP] were increased there was virtually no change in Ca\textsuperscript{2+}-sensitivity of the contractile apparatus and maximum Ca\textsuperscript{2+}-activated force was ~106 ± 1 % compared to control conditions. These results suggest that the initial improvement in performance observed with Cr supplementation is likely due in large part to direct effects of the accompanying decrease in myoplasmic ionic strength on the properties of the contractile apparatus.

Keywords: ergogenic aid, muscle contraction, fatigue
Introduction:
The ingestion of creatine (Cr) can result in improved performance during certain types of exercise (1, 4, 27, 29). It has been suggested that this ergogenic effect may be due to increased Cr phosphate (CrP) levels that thereby enhance the capacity to either resynthesise ATP (4) or to maintain ATP stores by reducing the loss of adenine nucleotides (1), or enhance acid-base buffering (27).

Water accumulation and increases in muscle mass are often reported as side effects following Cr administration. One study showed that muscle intracellular volume increased 2.3 and 3.1 % after 1 and 3 d Cr supplementation, respectively (32). Longer periods (5 d to 6 weeks) of Cr supplementation typically result in increases in fat free body mass of 1-2 kg (7, 28, 29). This weight gain equates to a 3.5-7 % percent increase in muscle water in a 70 kg person. Water moves into the muscle fibers following an increase in Cr content owing to the necessity to maintain osmotic balance. It has been suggested that since cellular swelling can act as an anabolic stimulator of protein synthesis (15), Cr supplementation may promote protein synthesis (32) and thereby muscle performance may be enhanced. Recently, however, it was demonstrated that muscle myofibrillar and sarcoplasmic protein synthesis were not different following Cr supplementation (20). This suggests that the effect of Cr ingestion on muscle mass is not through effects on muscle protein metabolism. Further consequences of the accumulation of cellular water with Cr have not been examined to date.
Studies have examined the effect of Cr supplementation on twitch and tetanic force production in human and rodent muscle (10, 16, 21, 23, 26, 30). In one study, no changes in peak tension, time to peak tension, or half-relaxation time during twitch or tetanic stimulations were observed in either soleus (SOL) or extensor digitorum longus (EDL) muscle in mice fed Cr compared to control mice (23). Other studies report similar findings of no improvements in twitch or tetanic force production or half-relaxation time following Cr supplementation in various muscles and species (10, 16, 21, 26, 30). Given that improvements in muscle performance of only a few percent can be very important, small changes need to be measurable. One aspect that should be noted in the animal studies is that unpaired data sets are used (10, 21, 23, 26, 30). This inevitably limits their ability to detect small differences. Given this, the findings of the lack of effect of Cr supplementation on twitch and tetanic force production may not be conclusive.

As mentioned, the benefit of Cr supplementation on muscle performance is usually thought to be due to an increased ability to resynthesise ATP via increased CrP stores. However, studies in humans report that the intramuscular CrP:Cr ratio actually falls following 5 d Cr supplementation (6, 14, 22, 28). This means that the Cr concentration is increased more than the CrP concentration, which in fact should hinder, not aid, the ability of the Cr kinase reaction to rephosphorylate any ADP to ATP. In view of this, it seems that an alternate mechanism is needed in order to explain the observed improvement in performance with Cr supplementation.
One possibility is that the Cr uptake into muscle directly or indirectly affects force production by the contractile apparatus. Previously it has been shown that the addition of 50 mM Cr to a control solution resulted in a decrease in maximum Ca\(^{2+}\)-activated force in rabbit single fibers (5, 11). Maximum Ca\(^{2+}\)-activated force was not affected, however, by the addition of 25 mM Cr (11). Those two studies represented a non-physiological change to the intracellular environment since, as stated above, when Cr enters the muscle fiber there is also an accumulation of water to maintain osmotic balance (32). The increase in cellular water is in fact a critical factor, as it means that the other cellular constituents are diluted and the ionic strength of the cytoplasm will decrease. There have been no studies to date that have measured the combined effects of Cr and the associated volume of water on the contractile apparatus in skeletal muscle.

Here, mechanically-skinned single fibers from rat skeletal muscle are used to measure the performance of the contractile apparatus (18, 25). Fibers from SOL and EDL muscles are studied in order to obtain fibers at the two extremes of fiber types (ie. predominantly slow oxidative and fast glycolytic, respectively) and therefore bracket the spectrum of fiber types. We are able to examine separately the direct effects on the contractile apparatus of increasing Cr plus decreasing ionic strength (mimicking the changes seen with acute Cr supplementation) and increasing Cr and CrP with no change in ionic strength (mimicking the changes seen with longer term Cr supplementation). Additionally, to determine if Cr \textit{per se} has an effect on the contractile apparatus we examine the effect of increasing Cr alone. Our approach allows the detection of even small differences (in the order of 1-2 %) in muscle performance because the same fiber is used for both control and test
experiments. Additionally, the cytoplasmic environment of the fiber can be readily manipulated to study individual factors or combinations of factors as desired. We hypothesised that an improvement in muscular performance following Cr supplementation might be due to a decrease in the ionic strength of the intracellular environment rather than any direct effect of Cr itself.

Methods:

Control solutions

All chemicals were obtained from Sigma (St Louis, Mo., USA). Heavily Ca$^{2+}$-buffered solutions of varying pCa ($\text{pCa} = -\log_{10} [\text{Ca}^{2+}]$) were prepared from two main solutions, high-relaxing (type I, pCa > 9) and high-activating (type II, pCa ~ 4.5), based on those described previously (9). The type I solution contained (in mM), 64 K$^+$, 100 Na$^+$, 25 EGTA, 1 free Mg$^{2+}$ (10.3 total Mg$^{2+}$), 9 total ATP, 19 Cr, 40 CrP, 1 inorganic phosphate (P$_i$) and 60 Hepes at pH 7.10 ± 0.01. The type II solution was made similarly, but with 24 mM total Ca$^{2+}$ (pCa ~ 4.5) and 9.25 total Mg$^{2+}$ to maintain the free Mg$^{2+}$ concentration at 1 mM. The type I and type II solutions were mixed to give a series of solutions with progressively increasing concentrations of Ca$^{2+}$ (pCa 6.7 – 4.5). These were then split in two to prepare the control and test solutions for each experiment, thereby producing two solution sets of matched pCa, pH and osmolality. In addition, Na$^+$-based Type I and II were prepared where all K$^+$ in the Type I and II solutions described above was replaced with Na$^+$. As for the K$^+$-based solutions, a set of matched solutions was prepared by mixing the Na$^+$ type I and type II solutions to give a series of solutions of increasing concentrations of Ca$^{2+}$.
**Mechanically-skinned fiber preparation**

Adult Long Evans hooded rats, aged 24-28 weeks, were killed by an overdose of halothane, as approved by the Animal Ethics Committee at La Trobe University. The EDL and SOL muscles were carefully removed, blotted dry on filter paper and pinned at resting length under paraffin oil. Single muscle fibers were dissected, mechanically skinned, mounted on a force transducer (AME875, SensoNor, Horten, Norway) and stretched to 120 % of their resting length. Each fiber was then placed into a 2 ml bath containing the high-relaxing solution (pCa>9) to equilibrate for 2 min before being activated in solutions of higher Ca$^{2+}$ concentration (see below). Experiments were performed at room temperature (24 ± 1°C).

**Ca$^{2+}$-activation of the contractile apparatus**

The force-pCa relationship for each fiber was determined by exposing the fiber to a sequence of solutions at progressively higher concentrations of Ca$^{2+}$, allowing the fiber to reach close to a steady-state force level in each solution before moving to the next. The final solution was the high-activating (pCa ~ 4.5) solution, and the force elicited in that solution was defined as the maximum Ca$^{2+}$-activated force. In each experiment the same fiber was used to measure one or more test conditions bracketed by control measurements. Between each of the activation sequences the fiber was relaxed in the high-relaxing solution (pCa >9) for 1 min. All results are on fibers where the decline in the maximum Ca$^{2+}$-activated force over the full sequence of controls and tests was less than 10 %.
**Experimental conditions**

The first set of experiments examined the effect of increasing Cr concentration on the force-pCa relationship of the contractile apparatus using the heavily Ca^{2+}-buffered solutions described above. Cr accumulation in muscle cells *in vivo* is accompanied by an increase in cellular water. This would result in osmolality being maintained and ionic strength being decreased (formal ionic strength, $\Gamma/2 = \frac{1}{2} \sum C_i Z_i^2$, where $C_i$ and $Z_i$ are the concentration and charge, respectively, of the $i^{th}$ ionic species). These conditions were mimicked in this first set of experiments. Control solutions contained 19 mM Cr and 40 mM CrP (CrP:Cr ratio = 2.1:1). In the test solutions, Cr was increased by 10 and 14 mM with concomitant decreases in the ionic strength and the concentration of other major constituents by 3.4 % (Cr10-Osm) and 5 % (Cr14-Osm), respectively. The final concentrations of Cr were approximately 28 and 32 mM in the Cr10-Osm and Cr14-Osm solutions, respectively. Details of the solutions are given in Table 1. Dilution of the solutions was achieved by the addition of water, the volume determined by the amount required to maintain osmolality of the solution following the addition of Cr. In the Cr10-Osm and Cr14-Osm solutions the ratio of CrP:Cr decreased from 2.1:1 to 1.4:1 and 1.2:1, respectively, similar to that that would be expected *in vivo* immediately following the accumulation of Cr into a muscle fiber. Additionally, the effect of a 14 mM increase in Cr alone (Cr14) was tested (ie. no accompanying addition of water and therefore no change in ionic strength). The final Cr concentration in this solution was 33 mM (see Table 1). In matched pairs of solutions, Cr14 solutions were prepared by adding 14 mM
Cr to one half of the heavily Ca\textsuperscript{2+}-buffered solutions of varying Ca\textsuperscript{2+} concentrations. The other halves of these solutions were used for the control conditions.

In the second set of experiments, the effect of increasing total Cr (TCr = CrP + Cr) by 6 mM (TCr6) and 9 mM (TCr9) plus water (ie. at constant ionic strength) was examined. These conditions mimicked the intracellular environment following longer term Cr supplementation where some of the Cr has been phosphorylated to CrP and ionic strength is largely restored. The TCr6 and TCr9 solutions were prepared by adding a volume of TCr stock solution ([CrP] = 82 mM, [Cr] = 55 mM, CrP:Cr = 1.5:1) to one half of the matched heavily Ca\textsuperscript{2+}-buffered solutions. The TCr stock solution was prepared in order to be able to make solutions with raised concentrations of Cr and CrP and with the final CrP:Cr ratio of approximately 2:1, similar to the control solutions. With this procedure sufficient water was added with the neutral Cr and charged CrP species, such that there was no difference in final ionic strength between the TCr6 and TCr9 solutions and the control solutions (see Table 1). Additionally, pCa, pH or osmolality (290 \(\pm\) 8 mosmol(kg solvent))\textsuperscript{-1}) of the final TCr6 and TCr9 solutions were unchanged compared to the matched control solutions. Details of the solutions are shown in Table 1. The CrP used was Na\textsubscript{2}CrP and so to ensure that the concentration of Na\textsuperscript{+} was the same between the control and TCr6 and TCr9 solutions, the same volume of the appropriate mix of Na\textsuperscript{+}-based type I and type II solutions was added to the matched control solutions.
**Analysis**

Each activation sequence from each fiber was analysed individually. For all sequences, the force achieved at each \( \text{Ca}^{2+} \) concentration was expressed relative to the maximum \( \text{Ca}^{2+} \)-activated force obtained for that sequence. The force-pCa data was then fitted with a Hill curve based on the equation below (GraphPad Prism 4, GraphPad Software Inc, San Diego CA, USA, 2003) to obtain the pCa\(_{50} \) (the \( \text{Ca}^{2+} \) concentration expressed in pCa units giving 50 % of maximum force) and the Hill coefficient (\( n_H \)).

\[
\text{The relative force, } F(p\text{Ca}) = \frac{1}{1 + 10^{(p\text{Ca} - p\text{Ca}_{50})n_H}}
\]

Results are expressed as means ± SEM of \( n \) observations. Statistical significance was determined at 95 % confidence level using two-tailed Student’s \( t \)-test for paired samples.

**Results**

In order to maintain cellular osmolality, the accumulation of Cr into muscle cells results in an accompanying increase in muscle water. A consequence of water being drawn into the cell is a concomitant decrease in the concentration of other cellular constituents and importantly, a decrease in the ionic strength of the myoplasm. The first part of the first experiment examined the effects of increasing both Cr concentration and water content on the contractile apparatus of mechanically-skinned single fibers from rat SOL and EDL muscle. Figure 1 shows the steady-state force response from a SOL fiber over a range of \( \text{Ca}^{2+} \) concentrations, using heavily \( \text{Ca}^{2+} \)-buffered solutions, when exposed to control and test (Cr10-Osm) solutions. Hence the same fiber was used as its own control. This can be seen in Figure 1 by the two Cr10-Osm force-pCa staircases bracketed by two control
staircases. For each fiber, the isometric force generated under both the control and test conditions was then plotted against pCa, and each individual force-pCa data set was fitted with a Hill curve. The Hill plots for the two control sequences and the two test (Cr10-Osm) sequences for the SOL fiber in Figure 1 are shown in Figure 2. The relative maximum Ca$^{2+}$-activated force in Cr10-Osm and Cr14-Osm and the mean change (Δ) in pCa$_{50}$ (the Ca$^{2+}$ concentration in pCa units giving 50 % of maximum force) and in the Hill coefficient ($n_H$) are given in Table 2. Compared to control conditions, maximum Ca$^{2+}$-activated force in the Cr10-Osm conditions increased to 106 ± 1 % in SOL fibers and 106 ± 2 % in EDL fibers. In the Cr14-Osm conditions, maximum Ca$^{2+}$-activated force increased to 104 ± 1 % and 105 ± 1 % in SOL and EDL, respectively, compared with control conditions. Compared to the addition of 10 mM Cr (Cr10-Osm), adding 14 mM Cr (Cr14-Osm) would result in a greater decrease in ionic strength of the solutions (see Table 1). It would be expected that the increase in maximum Ca$^{2+}$-activated force would be greater under the Cr14-Osm conditions, though in fact the increase in maximum force appeared to be slightly greater under Cr10-Osm compared to Cr14-Osm conditions (see Table 2). This small difference, however, was evidently due to the particular sub-population of fibers sampled because in each case when any given fiber was examined in both of the two test solutions (ie. Cr10-Osm and Cr14-Osm), maximum Ca$^{2+}$-activated force was in fact always greater in the Cr14-Osm solution compared with Cr10-Osm solution (+1.3 ± 0.4 %, p<0.05, n=7).

In both Cr10-Osm and Cr14-Osm solutions, the force-pCa relationship was shifted to the left (ie. shifted to a lower Ca$^{2+}$ concentration) in SOL and EDL fibers (Figure 2). The
mean ΔpCa50 was +0.044 ± 0.003 and +0.034 ± 0.005 in SOL and EDL, respectively with Cr10-Osm conditions and +0.061 ± 0.004 and +0.049 ± 0.009, respectively in Cr14-Osm conditions (Table 2). The Hill plot for SOL fibers became slightly less steep, as indicated by the small decrease in nH, (-1.4 ± 0.3 and -0.4 ± 0.1 for Cr10-Osm and Cr14-Osm, respectively). In EDL fibers, the steepness of the force-pCa relationship was not significantly affected in either solution (Table 2).

To determine if the effects seen in the Cr10-Osm and Cr14-Osm solutions were due to effects of Cr alone (rather than to the decrease in ionic strength) another condition was examined. In this case the concentration of Cr was increased by 14 mM (Cr14) without an accompanying increase in water (see Methods). Given that Cr entering a muscle cell will always take water with it, the Cr14 solutions do not mimic the physiological situation. The conditions nevertheless enabled the direct effects of Cr per se on the contractile apparatus to be examined. The associated 14 mosmol(kg solvent)1 increase in osmolality would not be expected to alter the contractile properties of the fibers (19). Importantly, there is no change in the ionic strength between the control and Cr14 solutions. As can be seen in Table 2, the effects of the Cr14 conditions were in the opposite direction to those seen in the Cr10-Osm and Cr14-Osm conditions. In both SOL and EDL, maximum Ca2+-activated force decreased to 97 ± 1 % compared to the control conditions and ΔpCa50 decreased slightly (–0.026 ± 0.004 and –0.018 ± 0.005 in SOL and EDL fibers, respectively, Table 2). There was no change observed in nH in either fiber type. These results show that Cr per se has a slightly inhibitory effect on Ca2+-activated force, which contrasts with the substantial increase in maximum force and Ca2+-
sensitivity observed when both Cr and water are present. Thus, the increase in Cr concentration was not the cause of the changes in the latter case.

Following short-term (5-10 d) Cr supplementation protocols, increases in intramuscular TCr stores are typically in the order of 15-25 % in human and rodent muscle (2, 3, 6, 12, 14, 22-24, 28). Once Cr enters the muscle fibers, it takes time for it to be phosphorylated to CrP (6, 12, 14, 22, 28). This results in there being an initial decrease in the CrP:Cr ratio following Cr supplementation, which is only restored towards original levels over longer periods of time. In the second set of experiments, solutions were prepared to mimic the myocellular environment following increases in both Cr and CrP contents and the re-establishment of the initial CrP:Cr ratio (and hence the ionic strength), as is sometimes reported to occur with longer term low dose Cr supplementation (6). Solutions were prepared with increases in TCr of either 6 mM (TCr6) or 9 mM (TCr9), and the final CrP:Cr ratios were 2:1 and 1.9:1, respectively, close to control conditions (see Table 1). These changes equated to 11 % and 15 % increases in TCr and therefore conservatively mimicked the range observed in vivo following Cr supplementation. The isometric force generated at different pCa values in a SOL fiber under both control and TCr6 conditions were fitted with Hill curves and are shown in Figure 3. The ΔpCa_{50}, Δn_H and the maximum Ca^{2+}-activated force in TCr6 and TCr9 for all fibers are given in Table 3. Maximum Ca^{2+}-activated force in the TCr6 conditions increased to 106 ± 1 % in SOL and EDL, and to 104 ± 1 % in SOL in TCr9 conditions. There was virtually no change in the pCa_{50} in TCr6 solutions, whilst in TCr9 solutions this increased slightly in SOL (+0.028 ± 0.002). The n_H became slightly shallower in SOL in both the TCr6 and TCr9
solutions (-0.4 ± 0.1 and -0.5 ± 0.1, respectively) and was not affected in EDL in TCr6 conditions (Table 3). Overall, these results indicate that when there is an increase in the contents of CrP and Cr some potentiating action remains even though the CrP:Cr ratio and the ionic strength were returned to original values. These effects are seen as an increase in maximum Ca\(^{2+}\)-activated force and a slight increase in Ca\(^{2+}\)-sensitivity of the contractile apparatus of the fibers.

**Discussion**

In this study we have used mechanically-skinned single fibers from rat SOL and EDL muscles to examine the individual direct effects on the contractile apparatus of increasing Cr, increasing Cr plus decreasing ionic strength, and increasing Cr and CrP with no change in ionic strength.

Using changes similar to those occurring in muscle with short-term Cr supplementation studies, the present study determined the effect of increasing Cr and decreasing ionic strength on the contractile apparatus of rat skeletal muscle fibers. We found that increasing the Cr concentration of a muscle fiber, along with an appropriate increase in cellular water to maintain osmolality, resulted in an increase in the Ca\(^{2+}\)-sensitivity as well as the maximum Ca\(^{2+}\)-activated force in both SOL and EDL fibers. When Cr was added to the muscle cytoplasmic environment without the addition of water, however, the changes were in the reverse direction. The major difference between these two conditions was the ionic strength, this being decreased when water was added (Cr10-Osm and Cr14-Osm compared to Cr14). These results show that there is actually a small detrimental
effect of Cr itself on the contractile apparatus of skeletal muscle, but that this is reversed when osmolality is maintained by the addition of water, and force responses are instead potentiated.

There have been a many studies published that have examined the effect of Cr supplementation on muscle performance and metabolism (2, 22, 27-29). In all cases attempts were made to increase intramuscular TCr stores by the oral administration of Cr. Whilst it is accepted that TCr stores are often increased, outcomes are perturbed by factors such as variations in initial TCr content (14) and fiber or muscle type (23, 24). Nevertheless, when TCr stores are increased in the order of 15 % or more, ergogenic effects are seen with certain exercise regimes (for review see 27). Improvements in muscle performance have been attributed to factors including an enhanced capacity to either resynthesise ATP via increased CrP levels, or to maintain ATP stores via a reduced loss of adenine nucleotides, or an enhanced acid-base buffering (27). Since the accumulation of Cr into a muscle fiber is accompanied by an increase in muscle cell water (32), there would in fact always be a decrease in cytoplasmic ionic strength following Cr supplementation. The accumulation of water in the muscle is reported as an increase in fat free body mass (32). This increase in fat free body mass is unlikely to be due to an increase in muscle protein given that myofibrillar and sarcoplasmic protein contents are not affected by Cr supplementation (20). We have shown that decreasing ionic strength by 3.4 or 5 %, equivalent to that estimated as a 1-2 kg increase in fat free body mass in a 70 kg man, results in an increase in the Ca\(^{2+}\)-sensitivity as well as the maximum Ca\(^{2+}\)-activated force in both EDL and SOL fibers from rat skeletal muscle. It
has previously been shown in mechanically-skinned single fibers from EDL and SOL muscle that a 40 % decrease in ionic strength resulted in an increased Ca\(^{2+}\)-sensitivity (+0.38 pCa units) and maximum Ca\(^{2+}\)-activated force (+20 %) of the fibers (8).

Consistent with this, in the present study the decrease in ionic strength was approximately 10-fold less (3.4 and 5 %) and the observed shift of +0.047 in the pCa50 about 10-fold smaller than that seen by Fink and co-workers (8). In other words, the changes found here are well accounted for by the known effects of changes in ionic strength on the contractile apparatus. The present study found an increase in Ca\(^{2+}\)-sensitivity (ΔpCa ~ +0.05, note this is in a log scale) of ~ 10 % and an increase of ~ 6 % in maximum Ca\(^{2+}\)-activated force. Such improvements in muscle performance could be highly beneficial in terms of an individual’s competitive outcome. These findings suggest that increasing Cr and water in muscle cells would result in two benefits: (i) an increase in tetanic peak force (produced by the increase in maximum Ca\(^{2+}\)-activated force) and (ii) an increase in Ca\(^{2+}\)-sensitivity (ie. at a given Ca\(^{2+}\) concentration more force would be produced, see lower part of Figure 2).

Previous studies that have measured the effects of Cr supplementation on tetanic force production have found no effect in human and rodent models (10, 16, 21, 23, 26, 30). However, a number of limitations existed in those studies. Firstly, the protocols relied on the individual responding to the Cr ingestion by increasing Cr stores in the muscle. Intracellular TCr was measured in only some of the studies (23, 26), and so it is uncertain if the Cr concentration was indeed increased in the others (10, 16, 21, 30). It is known that in humans there is a variable response by individuals to Cr supplementation (14) and
in animals the uptake of Cr into muscle seems to be fiber type dependent (23, 24). A second major limitation in the animal studies was the necessity to use unpaired sampling sets making it hard to see small differences (10, 21, 23, 26, 30). In rats, 10 d Cr supplementation resulted in a tendency for tetanic force to increase when normalised to weight ($501 \pm 49 \text{ g.g}^{-1}$ and $547 \pm 16 \text{ g.g}^{-1}$ in control and Cr fed rats, respectively) of SOL muscle (30). Whilst those results were not significant, the results from the present study indicate that increases in muscle Cr and water produce only a small increase in maximum Ca$^{2+}$-activated force, which would translate into tetanic force being augmented.

During fatigue, sarcoplasmic reticulum Ca$^{2+}$ release may be reduced, resulting in lower cytoplasmic Ca$^{2+}$ concentrations. In the present study we saw an increase in Ca$^{2+}$-sensitivity when the Cr concentration and water content are both raised (eg. Cr10-Osm conditions) compared with control conditions. These findings suggest that more force would be produced at the lower Ca$^{2+}$ concentrations occurring during fatigue. This can be seen in Figure 2 where for a given concentration of Ca$^{2+}$, more force is produced. For example, at pCa 5.78, ~19 % of maximum force is produced under control conditions and ~35 % of maximum force is produced in the Cr10-Osm conditions (ie. almost double the force).

To determine if the benefits we observed in Cr10-Osm and Cr14-Osm could be attributable to Cr per se we investigated the effect of increasing the concentration of Cr by 14 mM in the cytoplasmic environment without altering the other constituents or total water. The 14 mM increase in Cr concentration gave a final concentration of 33 mM Cr
(see Table 1). Whilst this manipulation would not occur in a muscle cell (as osmotic equilibrium is not maintained), it enabled examination of the effect of Cr per se on muscle contractile apparatus performance. In this set of experiments we found the effects to be in the opposite direction to those of the Cr plus water experiments (Cr10-Osm and Cr14-Osm). Previous work reported a decrease in Ca\(^{2+}\)-sensitivity and maximum Ca\(^{2+}\)-activated force in rabbit fast twitch muscle when the Cr concentration was 50 mM compared to 0 mM (5, 11). Interestingly, no difference was detected with 25 mM Cr compared to 0 mM Cr (11). These and our data suggest that a small deleterious effect on Ca\(^{2+}\)-sensitivity and maximum Ca\(^{2+}\)-activated force might occur when Cr concentrations are greater than ~ 33 mM. The improvements in Ca\(^{2+}\)-sensitivity and maximum Ca\(^{2+}\)-activated force when an increase in Cr is accompanied by the accumulation of water to maintain osmotic balance are clearly not the consequence of increasing Cr stores per se. It is unclear why Cr at concentrations above 33 mM has a deleterious effect on maximum Ca\(^{2+}\)-activated force. It may be because the increase in free Cr inhibits the ability of the Cr kinase to rephosphorylate any ADP to ATP in localised regions of high ATP usage within the fiber. We note, however, that previous work reported that even when Cr kinase was inactivated, 50 mM Cr was deleterious to force production (5).

Studies using maintenance doses of Cr supplementation for 6-8 weeks have reported the CrP:Cr ratio either returning to pre-supplementation levels (6), or remaining lower than the initial level (28). In the present study when both the Cr and CrP contents were increased (TCr6 and TCr9), the ionic strength and CrP:Cr ratio were set at levels similar to the control conditions. It was found that the apparent Ca\(^{2+}\)-sensitivity was virtually
unchanged with 6 mM additional TCr, but increased slightly with 9 mM additional TCr (see Table 3). Importantly, maximum Ca\(^{2+}\)-activated force increased to 104-106 % of that in the control solution. Given that ionic strength was not different in the control and test solutions, this improvement in maximum force must be due to some other factor. One possibility is that it is the result of improved buffering of ATP in localised regions of high ATP usage, in particular in the vicinity of the myosin heads during prolonged activation.

Even though the ratio of CrP:Cr is no different in the TCr6 and TCr9 solutions than it is in the control solution, the absolute amounts are increased, and this would mean that there would be increased diffusion of CrP into any local regions where it had been depleted. Thus, the TCr6 and TCr9 solutions would help ensure better buffering of CrP and ATP in localised regions where ATP is being used at a high rate (17), and where the build-up of ADP and other metabolites could be retarding force production. This effect could also be even more important during times of increased ATP usage with active shortening of the fibre (31). This could explain the improvement in muscle peak torque production in humans following the ingestion of Cr compared to the ingestion of a placebo (13).

In summary, the present study suggests that following short-term Cr supplementation the decrease in ionic strength that accompanies the accumulation of intramuscular water will result in beneficial effects on the contractile apparatus. These effects are unlikely to be due to the increase in Cr alone, which in fact seems to be detrimental to force production. Evidently, the deleterious effects on muscle contractile properties of increased Cr per se are normally completely offset by the benefits associated with the accompanying
decrease in ionic strength that occurs *in vivo*. These effects offer an explanation for the
erogenic effect of Cr supplementation that is observed even before intramuscular CrP
content has increased substantially. Following longer term Cr supplementation, when
both the CrP:Cr ratio and the ionic strength have returned towards the pre-
supplementation values, a beneficial effect on maximal force production persists. This
effect is possibly due to the increase in intramuscular CrP content and the associated
enhancement in ATP rephosphorylation capacity in localised regions of high ATP usage.

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29. **Volek JS, Duncan ND, Mazzetti SA, Staron RS, Putukian M, Gomez AL, Pearson DR, Fink WJ, and Kraemer WJ.** Performance and muscle fiber adaptations to


Table 1. Final creatine (Cr) and Cr phosphate (CrP) concentration, CrP:Cr ratio and formal ionic strength (Γ/2) of solutions. Details of how solutions were prepared are in the Methods. The osmolality of the solutions were ~ 290 mosmol(kg solvent)$^{-1}$ except for Cr14 which was 14 mosmol(kg solvent)$^{-1}$ greater. Note that Γ/2 of the Cr10-Osm and Cr14-Osm solutions are 3.4 % and 5 % lower than the other solutions.

Table 2. Summary of effects of addition of water and creatine (Cr), or addition of Cr alone, on the contractile apparatus properties.

Data are given as mean ± SEM for the relative maximum Ca$^{2+}$-activated force (maximum force expressed as a percentage) and the changes (Δ) in pCa$_{50}$ and the Hill coefficient ($n_H$), for the steady-state force responses in test solutions compared to the bracketing control response (before and after tests). Compared to the control solution, test solutions included the addition of 10 mM Cr (Cr10-Osm) or 14 mM Cr (Cr14-Osm) plus the addition of water to give either a 3.4 or 5 % dilution, respectively, to maintain osmolality, or the addition of 14 mM Cr (Cr14) alone. The mean control values for pCa$_{50}$ and $n_H$ were 5.783 ± 0.029 and 5.4 ± 0.4, respectively, for SOL (n = 13) and 5.781 ± 0.046 and 7.2 ± 0.7, respectively, for EDL (n = 13) fibers. * significant difference between the test condition and the bracketing control responses using Student’s paired two-tailed t-tests (p<0.05).
Table 3. Summary of effects of addition of 6 mM or 9 mM total creatine at constant CrP:Cr ratio on the contractile apparatus properties.

Data are given as mean ± SEM for the relative maximum Ca^{2+} activated force (maximum force expressed as a percentage) and changes (Δ) in pCa_{50} and Hill coefficient (n_{H}) for the steady-state force responses in test solutions compared to the bracketing control response (before and after test). Compared to control solutions, test solutions included the addition of 6 mM or 9 mM total creatine (TCr = CrP + Cr, final CrP:Cr = 1.9). The mean control values for pCa_{50} and n_{H} were 6.022 ± 0.009 and 3.6 ± 0.2, respectively, for soleus (n = 9) and 5.975 ± 0.034 and 4.2 ± 0.2, respectively, for EDL (n = 6) fibers. * significant difference between the test condition and the bracketing control responses using Student’s paired two-tailed t-tests (p<0.05).
**Figure 1:** Force-pCa behaviour for a soleus fiber exposed to control and test (increased contents of creatine and water) conditions. A skinned soleus fiber was exposed to stepwise increasing concentrations of Ca^{2+} (upwards arrows, ↑, denote [in pCa units] 1 [≥9, no force], 2 [6.45], 3 [5.95], 4 [5.78], 5 [5.64], 6 [5.50], 7 [4.5, maximum Ca^{2+}-activating solution]) under control conditions (---) and test conditions (Cr10-Osm, ==, 10 mM creatine plus addition of water to maintain osmolality which gives a 3.4 % dilution of other constituents). The force produced is shown for two control activation sequences bracketing two Cr10-Osm sequences. The upper dashed line (A ----) indicates the steady-state force response at maximum Ca^{2+}-activated force and the lower dashed line (B ----) indicates the steady-state force response at pCa 5.64. In both traces it can be seen that more force was produced in the two Cr10-Osm responses compared with their control responses. There was a greater difference between the two conditions at pCa 5.64 (B) than at maximum Ca^{2+}-activated force (A) indicating an increase in Ca^{2+}-sensitivity.

**Figure 2:** Effect on contractile apparatus of increasing creatine concentration by 10 mM plus the addition of water resulting in a 3.4 % decrease in ionic strength. Force-pCa relationships for the soleus fiber shown in Figure 1 exposed to a series of solutions with increasing concentrations of Ca^{2+}, under control conditions (■) and test conditions (Cr10-Osm, 10 mM creatine plus 3.4 % dilution, □) and expressed relative to the average maximum Ca^{2+}-activated force in the control solutions. For both conditions there are two sets of measurements that were very similar and overlies each other in the plot. The pCa_{50} (Ca^{2+} concentration in pCa units at which half maximum force is reached) and Hill coefficient (n_H) for each response are given below the Figure. The Cr10-Osm
conditions resulted in a shift to the left in the force-pCa curve compared to the control curve, indicating an increase in Ca\(^{2+}\)-sensitivity, ie. a greater relative force was elicited at the same absolute Ca\(^{2+}\) concentration. Additionally, the maximum Ca\(^{2+}\)-activated force produced in Cr10-Osm conditions was higher than that in the control conditions.

**Figure 3**: Effect on contractile apparatus of a 6 mM increase in total creatine concentration with CrP:Cr ratio and ionic strength kept approximately constant. Force-pCa relationships for a soleus fiber exposed to a series of solutions with increasing concentrations of Ca\(^{2+}\), under control conditions (■) and test conditions (addition of 6 mM total creatine, TCr6, final CrP:Cr = 1.9:1, □) and expressed relative to the average maximum Ca\(^{2+}\)-activated force in the control conditions. The pCa\(_{50}\) and Hill coefficient \((n_H)\) for each case are given below the Figure. Maximum Ca\(^{2+}\)-activated force was higher in TCr6 compared to control conditions. Comparing control and TCr6 conditions, there is virtually no change in the Ca\(^{2+}\)-sensitivity, ie. a similar relative force was elicited at the same absolute Ca\(^{2+}\) concentration.
Table 1. Final creatine (Cr) and Cr phosphate (CrP) concentration, CrP:Cr ratio and formal ionic strength (Γ/2) of solutions. Details of how solutions were prepared are in the Methods. The osmolality of the solutions were ~ 290 mosmol(kg solvent)$^{-1}$ except for Cr14 which was 14 mosmol(kg solvent)$^{-1}$ greater. Note that Γ/2 of the Cr10-Osm and Cr14-Osm solutions are 3.4 % and 5 % lower than the other solutions.

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**Table 2. Summary of effects of addition of water and creatine (Cr), or addition of Cr alone, on the contractile apparatus properties.**

Data are given as mean ± SEM for the relative maximum Ca^{2+}-activated force (maximum force expressed as a percentage) and the changes (Δ) in pCa50 and the Hill coefficient (nH), for the steady-state force responses in test solutions compared to the bracketing control response (before and after tests). Compared to the control solution, test solutions included the addition of 10 mM (Cr10-Osm) or 14 mM Cr (Cr14-Osm) plus the addition of water to give either a 3.4 or 5 % dilution, respectively, to maintain osmolality, or the addition of 14 mM Cr (Cr14) alone. The mean control values for pCa50 and nH were 5.783 ± 0.029 and 5.4 ± 0.4, respectively, for SOL (n = 13) and 5.781 ± 0.046 and 7.2 ± 0.7, respectively, for EDL (n = 13) fibers. * significant difference between the test condition and the bracketing control responses using Student’s paired two-tailed t-tests (p<0.05).
Table 3. Summary of effects of addition of 6 mM or 9 mM total creatine at constant CrP:Cr ratio on the contractile apparatus properties.

Data are given as mean ± SEM for the relative maximum Ca^{2+} activated force (maximum force expressed as a percentage) and changes (Δ) in pCa_{50} and Hill coefficient (n_H) for the steady-state force responses in test solutions compared to the bracketing control response (before and after test). Compared to control solutions, test solutions included the addition of 6 mM or 9 mM total creatine (TCr = CrP + Cr, final CrP:Cr = 1.9). The mean control values for pCa_{50} and n_H were 6.022 ± 0.009 and 3.6 ± 0.2, respectively, for soleus (n = 9) and 5.975 ± 0.034 and 4.2 ± 0.2, respectively, for EDL (n = 6) fibers. * significant difference between the test condition and the bracketing control responses using Student’s paired two-tailed t-tests (p<0.05).
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