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Comparison of the effects of inorganic phosphate on caffeine-induced Ca^{2+}-release in fast- and slow-twitch mammalian skeletal muscle.

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Running Title

Effects of phosphate in fast- and slow-twitch muscle

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Abstract

We compared the effects of 50 mM Pi on caffeine-induced Ca^{2+} release (Caff-ICR) in mechanically-skinned fast-twitch (FT) and slow-twitch (ST) skeletal muscle fibers of the rat. The time integral (area) of the Caffeine response was reduced by ~57% (FT) and ~27% (ST) after 30 s exposure to 50 mM Pi in either the presence or absence of creatine phosphate (to buffer ADP). Differences in the SR Ca^{2+} content between FT and ST fibers (~40% vs. 100% SR Ca^{2+} content (pCa 6.7), respectively) did not contribute to the different effects of Pi observed above; under-loading the SR of ST fibers so that the SR Ca^{2+} content approximated that of FT fibers resulted in an even smaller (~21%), but not significant reduction in Caff-ICR by Pi. The observed differences between FT and ST fibers described above could arise from fiber type differences in the ability of the SR to accumulate Ca^{2+}-Pi precipitate. To test this, fibers were Ca^{2+} loaded in the presence of 50 mM Pi. In FT fibers the maximum SR Ca^{2+} content (pCa 6.7) was subsequently increased by up to 13 times of that achieved when loading for 2 mins in the absence of Pi. In ST fibers, the SR Ca^{2+} content was only doubled. These data show that Ca^{2+} release in ST fibers was less affected by Pi than FT fibers and this may be due to a reduced capacity of ST SR to accumulate Ca^{2+}-Pi precipitate. This may account in part for the fatigue-resistant nature of ST fibers.

Keywords:

Fatigue

Ca^{2+} precipitation

Excitation-contraction coupling

Introduction

In vertebrate skeletal muscle, the generation of an action potential at the neuromuscular junction ultimately leads to muscle contraction. There are several important intermediary steps linking these two events, which together describe a process that is broadly termed Excitation-Contraction (E-C) coupling. More specifically, the action potential rapidly spreads across the surface membrane of a muscle fiber and into the transverse tubular (t-) system to elicit a depolarization that is subsequently sensed by specialized voltage-dependent Ca^{2+} channels termed the Dihydropyridine Receptors (DHPRs). The DHPRs in turn activate closely associated Ca^{2+} channels (termed the Ryanodine Receptors, RyRs) located in the terminal cisternae of the Sarcoplasmic Reticulum (SR). Calcium is subsequently released into the cytoplasm where it then binds to the Ca^{2+} regulatory system and initiates contraction (28).

Muscle contraction is an energy intensive process. The basic energy currency of a muscle cell is ATP, which is formed intracellularly from the degradation of a number of fuels (e.g. glucose) (19). The importance of ATP to the many steps in E-C coupling ensures that the cytoplasmic [ATP] remains relatively well buffered at high concentrations (~7-8 mM per litre of cytoplasmic water) (1). This is accomplished by the generation of ATP through both aerobic and anaerobic processes and by the rapid hydrolysis of creatine phosphate (CP) (19). However, with repeated and prolonged activity, skeletal muscle force output declines with time. This phenomenon is termed fatigue (12).

Fatigue is more profound in the FT fibers than in the ST fibers, leading to the latter fiber type being often referred to as fatigue resistant. The difference in the fatigueability of these two fiber types seemingly arises from the different capacity of these fiber types to maintain the ATP pool. In FT fibers, during prolonged high intensity activity, ATP synthesis can be limited. Consequently, as ATP catabolism exceeds synthesis, a rapid and large increase in the concentrations of ATP metabolites, such as ADP (~1-2 mM) and Pi (30-50 mM), occurs. The increase in Pi has been strongly implicated in metabolic fatigue in FT fibers (15).

In FT fibers, Pi has been shown to directly inhibit maximum Ca^{2+}-activated force and the Ca^{2+}-sensitivity of the contractile apparatus (6, 29). Pi has also been implicated in the sudden rapid decline in Ca^{2+} release from the SR seen in the later stages of metabolic fatigue. Fryer et al. (15) first suggested that Pi enters the SR whereby it precipitates with Ca^{2+} to limit the free Ca^{2+} available for release. Posterino and Fryer (33) later showed that the entry of Pi into the SR is seemingly passive (ATP independent) and it is now thought that Pi enters the SR through a small conductance chloride channel (23). Other studies using single intact fibers further confirmed the importance of Pi in the failure of Ca^{2+} release and fatigue in FT fibers (21, 39). However, previous studies by Duke and Steele (7, 8) suggested that a failure in ADP buffering may play an important role in the apparent Pi induced failure of Ca^{2+} release seen in past skinned fiber studies. Many previous skinned fiber studies examined Pi in the absence of CP and thus, seemingly failed to adequately buffer ADP, which could potentially reverse the flow of Ca^{2+} through the SR Ca^{2+}-ATPase. Recently, however, Dutka et al. (11) showed that Pi could reduce the available Ca^{2+} in the SR for release in FT even when ADP was well buffered with CP.

Although ST fibers have a higher aerobic capacity than FT fibers, during repeated and prolonged activity, the cytoplasmic [Pi] can rise to similar levels as that seen in FT fibers (20-30 mM) (22). However, there has been no previous examination of the effects of Pi on Ca^{2+} release from the SR in ST fibers and therefore, its role in fatigue in these fiber types is not known. A recent study by Macdonald & Stephenson (27) showed that Ca^{2+}-handling in ST fibers is significantly less sensitive to rises in cytoplasmic ADP than FT fibers, suggesting that the underlying fatigue resistance of these fiber types may be a reduced sensitivity to ADP, amongst other metabolites thought to be involved in fatigue.

Therefore, the aim of this study was to compare the effects of Pi in both FT and ST skeletal muscle fibers in order to better understand the mechanisms that underlie the differences in fatigueability of these two fiber types. We used mechanically-skinned fibers in this study because this preparation allows both the SR Ca^{2+} content and the cytoplasmic [Pi] to be tightly controlled. We examined the effects of Pi in both fiber types in the presence and absence of CP, to identify any possible effects of ADP on Ca^{2+} leak from the SR due to possible pump reversal. Furthermore, because skinned fibers are effectively an open system (due to the removal of the surface membrane), the SR Ca^{2+} content may fluctuate due to Ca^{2+} movements into and out of the fiber that are independent of any effect of Pi. Consequently, this may influence observed results. Therefore, we also examined the effects of Pi in a closed system using paraffin oil as a barrier to limit exogenous Ca^{2+} movements and ensure the SR Ca^{2+} content remained constant, with the exception of any effect of Pi. We show for the first time that even at elevated Pi levels that approximate the most severe state found in FT fibers, SR Ca^{2+} release following Pi exposure was reduced more substantially

(by ~2 fold) in FT than ST fibers, indicating that ST fibers are more resistant to elevated Pi levels. Furthermore, this difference in the effects of Pi on Ca^{2+} release appears to be related to a difference in the ability to accumulate Ca^{2+}-Pi precipitate in these fiber types. Preliminary data has been previously reported in abstract form (32).

Methods

Animals and single fiber isolation

In accordance with the University of Adelaide animal ethics guidelines, male Hooded-Wistar rats (4-6 months age) were killed by asphyxiation with CO_2. Whole extensor digitorum longus (EDL) and soleus muscles were then dissected from the hind limb and pinned under paraffin oil at resting length. Using a dissecting microscope (Lieca), single muscle fibers were isolated and then mechanically-skinned with a pair of fine jewellers forceps (Dumont No. 5). One end of the fiber was subsequently tied with fine suture silk (Deknatel 10/0) to a force transducer (AE-800, Memscap artno, Norway; resonance frequency with stainless steel pin attached of >1 kHz) and the other end of the fiber clamped with a fixed pair of forceps. The resting length of the fiber was then measured before the fiber was stretched a further 20% (to bring the sarcomere length within the optimal force producing range (~3.1-3.2μM)) and the diameter of the fiber was then measured. The skinned fiber was subsequently immersed for 2 min. in potassium (K^+-) hexamethylene-diamine-tetraacetic acid (K-HDTA) solution that mimics the normal cytoplasmic milieu (see below).

Solutions

All chemicals were purchased from Sigma. The composition of skinned fiber solutions used is shown in Table 1. The K-HDTA solution was used to generate several solution types listed in Table 1. To maintain close to normal ionic strength and osmolality in solutions containing phosphate, HDTA was replaced with Pi at a ratio of 1:1.3. Osmolality was measured with a Roebling Automatic osmometer kindly lent to us by Professor Richard Ivell. The osmolality of the standard K-HDTA

solution was 295 ± 5 mosmols kg^{-1} while 50 mM P_i solutions varied by no more than ±10 mosmols kg^{-1}.

<Insert Table 1 here>

Experimental protocols

Estimation of releasable SR Ca^{2+}: A qualitative assessment of the amount of releasable Ca^{2+} from the SR of skinned fibers was determined by examining changes in the time integral (area) of the caffeine-induced force response elicited by exposure to the Caffeine solution (see Table 1). The area of the caffeine-induced force transient has been previously shown to be directly proportional to the SR Ca^{2+} content over a range of SR [Ca^{2+}] (33, 37). Figure 1 shows the relationship between the area of the caffeine-induced force response versus the length of time fibers were loaded with Ca^{2+}, in both FT and ST fibers. We used a correction method of Macdonald & Stephenson (26) to determine the force transient that would otherwise normally fall below a detectable range due the presence of 1 mM EGTA in the Caffeine solution in order to ascertain the 0 time point. In this instance, no correction was needed for ST fibers while all responses in FT fibers were corrected by adding 10% to the measured force transient consistant with previous work (37). It can be seen that there is a relatively linear relationship between the area versus the load time for both FT and ST fibers over a range of load times. In both fiber types the area was saturated by 2 mins of continuous loading at pCa 6.7 with longer load times (not shown) having no additional affect on the area in either fiber type.

<Insert Figure 1 here>

Initially, freshly skinned fibers were equilibrated in a K-HDTA solution (0.025 mM EGTA) for 2 mins and then briefly equilibrated in the wash solution (1 mM EGTA, 6

s) before the SR was emptied of Ca^{2+} using the Caffeine solution (30 mM caffeine, 0.02 mM free Mg^{2+}, 1 mM EGTA, 1 min; see Table 1). The Caffeine solution has been previously shown to rapidly empty the SR of all stored Ca^{2+} (16). The force response recorded initially (after the 2 min. equilibration above) was indicative of the endogenous SR Ca^{2+} content of the fiber. However, in many instances neither FT nor ST muscle fibers provided a measurable endogenous force response and possible reasons for this have been detailed by Trinh & Lamb (37). We know that the relative endogenous SR Ca^{2+} content of FT and ST fibers equates to an SR that is approximately $1/3^{rd}$ full and maximally loaded, respectively (16). Consequently, using the previously determined maximum Ca^{2+} content for each fiber type (see Figure 1) fibers were washed for 1 min in the Wash solution (see Table 1) and then re-loaded with Ca^{2+} using the Load solution (pCa 6.7, 1 mM total EGTA; see Table 1) for 30 s in FT fibers and for 2 min in ST fibers to mimic the different endogenous SR capacities; for ST fibers the SR was essentially fully loaded after ~ 30-40 s (see Figure 1) but we extended this to 2 min. to ensure uniform loads between all fibers. Fibers were then washed briefly in the Wash solution (10 s) before the amount of Ca^{2+} loaded into the SR was redetermined by exposure to the Caffeine solution. The area of the force response elicited was a measure of the total loaded Ca^{2+} after the 30 s and 2 min. loads indicated above. The peak force response to Caffeine as a percentage of the maximum Ca^{2+} activated force was 53 ± 6% (n=19) and 64 ± 5% (n=29) for FT and ST fibers, respectively. This indicated that the force responses to the Caffeine solution were submaximal.

Phosphate experiments: After the SR of single fibers was reloaded with Ca^{2+} to re-establish the near endogenous Ca^{2+} content (see above), fibers were then exposed to a

Wash solution (1 total EGTA) for 10 s (to stop further Ca^{2+} uptake) and then a further 16-36 s in the same solution before the SR was depleted of Ca^{2+} using the Caffeine solution. The caffeine-induced force response subsequently elicited was indicative of the Ca^{2+} content remaining in the fiber after equilibration for various times in the Wash solution that contained 1 mM EGTA. Under these conditions, the SR is expected to leak (loose) Ca^{2+}. After a 30 s equilibration (maximum equilibration period), we estimated this to be ~30% of the initial loaded Ca^{2+} content in FT fibers and 40% of the loaded Ca^{2+} in ST fibers. This is similar to previously reported estimates by others (2, 34). This response formed the control response with which subsequent caffeine-induced force responses following Pi exposure were compared to. Control responses were repeated twice to ensure reproducibility. Subsequently, fibers were again reloaded with Ca^{2+} in the Load solution, immersed briefly (6 s) in the Wash solution (to stop Ca^{2+} uptake) and then exposed for 10-30 s in a solution containing either 50 mM Pi and 0 mM CP (50Pi/0CP solution) or 50 mM Pi and 10 CP (50Pi/10CP solution) (see Table 1). Fibers were then washed in a Wash solution for 10 s to completely remove Pi from the fiber before being exposed to the Caffeine solution to ascertain the amount of releasable Ca^{2+} present within the SR. Fibers were then washed for 1 min (Wash solution) and the load-deplete protocol repeated again in the absence of Pi exposure. The area of the caffeine-induced force response elicited after Pi treatment was compared to the average control responses obtained before and after Pi treatment. Providing that the response to caffeine for bracketed controls remained relatively reproducible, the effect 50 mM Pi exposure in both the presence and absence of CP was examined in the same fiber.

Because the SR looses Ca^{2+} when allowed to leak in the Wash solution for various times, the endogenous content may not be well represented (fibers end up underloaded). Nevertheless, it was still expected that the SR contained a proportionately larger Ca^{2+} store in ST fibers than FT fibers after the load leak protocol described above, consistent with the differences in SR contents between the fiber types. Therefore, in some experiments, to ensure that the SR contents remained close to their original endogenous levels, both FT and ST fibers were equilibrated in paraffin oil (20 s) following a 10 s exposure to the Wash solution (control conditions) or equivalent 50Pi/0CP solution to limit the amount of Ca^{2+} leaked from the SR over this time (30 s). Under control conditions, there was <10% reduction in the SR Ca^{2+} content indicating that paraffin oil acted as an effective barrier to the movements of Ca^{2+} into and out of the fiber.

In other experiments (see results), we were also interested in examining the effect of approximately matching the SR Ca^{2+} contents of FT and ST fibers on the effect of a 30 s exposure to Pi on Ca^{2+} release. In this instance, ST fibers were Ca^{2+}-loaded for only 20 s and 30 s to bring the proportional SR Ca^{2+} content closer to that of FT fibers. From Figure 1 it can be seen that even after a 20 s load, the SR Ca^{2+} content of ST fibers was still large (~50% of its maximum Ca^{2+} content). Loading for shorter periods was deemed too inconsistent and was subsequently not done. Nevertheless, this load period reduced the total SR Ca^{2+} content and brought fibers closer to the range typically seen in FT fibers. Experiments were otherwise conducted as described above.

Pi-assisted SR Ca^{2+} loading: One way to indirectly determine the total capacity of the SR to store Ca^{2+}-Pi (and or Ca^{2+}) in both FT and ST fibers is to examine the ability of the SR to load Ca^{2+} in the presence of Pi. If the SR is permeable to Pi, then Ca^{2+} can be continuously loaded into the SR indefinitely until either the SR structure physically impedes further loading or the SR ruptures. This is possible because Ca^{2+} and Pi seemingly accumulate in the SR with a 1:1 stiochiometry (8, 15). Therefore, as Pi enters the SR the free Ca^{2+} in the SR lumen falls thus, the SR appears empty and Ca^{2+} uptake continues. The subsequent area of the response to Caffeine should then give a reasonable indication of the amount of Ca^{2+}-Pi stored. However, one would expect this to be an underestimate given that not all of the stored Ca^{2+} precipitated with Pi would be liberated from the SR over the course of the Caffeine stimulus thus, limiting the area of the Caffeine response.

Skinned fibers from both FT and ST muscles were initially Ca^{2+} loaded in the Load solution for 2 mins to saturate the SR with Ca^{2+}. Following a brief wash (10 s) in the Wash solution, the total SR Ca^{2+} content was determined by subsequently emptying the SR with the Caffeine solution. Fibers were then washed for 1 min in the Wash solution and then reloaded with Ca^{2+} for 2-5 mins in a modified 50Pi/10CP solution containing 1 mM total EGTA, pCa 6.7 (similar to the Load solution). The fiber was then washed for 10 s before the SR was again emptied of Ca^{2+} with the Caffeine solution. The area of the response to caffeine was then normalized to the area of the caffeine-induced force response obtained after 2 min loading in the absence of Pi.

Muscle Fiber typing: The soleus and EDL muscles predominately contain one major fiber type (slow and fast, respectively). However, there is a small proportion (~10%)

of the opposite fiber type in each muscle group. To confirm that selected skinned fibers were either of the fast or ST type we exposed fibers to a strontium-based EGTA solution (pSr 5.4, see Tale 1) at the end of each experiment. Based on the ~10-fold difference in the sensitivity of FT and ST muscle fibers to Sr^{2+} activation, we were able to confirm the fiber type of each fiber tested (31).

Statistical Analysis.

All data is presented as mean ± SE unless otherwise indicated. Data was compared using either a one-way or two-way ANOVA where appropriate, and considered significant if P was <0.05.

Results

Effects of Pi on caffeine induced Ca^{2+} release in FT fibers in the presence and absence of creatine phosphate.

We previously reported that Pi equilibrated in the absence of CP substantially reduced Caff-ICR in FT fibers consistent with the formation of a Ca^{2+}-Pi precipitate in the SR (15, 33). However, Duke and Steele (8) had attributed this reduction in Caff-ICR to a leak of Ca^{2+} from the SR arising from the absence of CP rather than an effect of Pi on free SR luminal Ca^{2+} *per se*. Thus, in the first instance, we were interested in comparing the effects of Pi equilibration in the presence and absence of 10 mM CP. Figure 2 shows a typical force trace obtained in a FT muscle fiber. It can be seen that neither the presence nor absence of CP during equilibration in 50 mM Pi markedly altered the ability of Pi to reduce Ca^{2+} release and peak force to caffeine exposure. In both instances the area of the caffeine-induced force response following Pi treatment was greatly reduced (by ~72% and 69% of bracketing controls, respectively).

<Insert Figure 2 here>

Figure 3 shows the mean data for these experiments in FT fibers treated with 50 mM Pi for between 10 s and 30 s. It can be seen that the area of the caffeine-induced force response was greatly reduced even after a 10 s exposure to 50 mM Pi, irrespective of the presence of CP (see Figures 3 A and B). Comparison of the mean areas for FT fibers across all the time points (10 s to 30 s) in the presence and absence of CP showed no significant difference between points (one-way ANOVA, $P>0.05$). Furthermore, the rapid time dependence and the extent of the reduction in force after

Pi treatment were not different from that previously reported by Posterino & Fryer (33).

<Insert Figure 3 here>

Effects of Pi on caffeine induced Ca^{2+} release in ST fibers in the presence and absence of creatine phosphate.

There has been no previous report examining the effects of phosphate on Ca^{2+}-handling in ST muscle. ST muscle is often referred to as fatigue resistant although the underlying mechanisms for this resistance have not been fully examined. Figure 4 shows a typical raw data trace examining the effects of 50 mM Pi exposure on a ST fiber in the presence and absence of CP. Surprisingly, exposure to Pi for 30 s only depressed the area of the subsequent caffeine-induced force response by ~21% in this fiber (contrast with the effect of Pi observed in the FT fiber in Figure 2). This indicated that Pi had a much smaller effect on Ca^{2+} release in ST fibers. Furthermore, the reduction in area was not altered by the presence of 10 mM CP in the Pi equilibration solution ($P>0.05$, one way ANOVA).

<Insert Figure 4 here>

Figure 3 also illustrates the mean ST data showing the effect of Pi on caffeine induced Ca^{2+} release in the absence (Figure 3A) and in the presence (Figure 3B) of CP for different exposure times (10 s to 30 s). It can be seen that the effect of phosphate on the area of the caffeine response was initiated rapidly (within 10 s) but then did not change with longer exposure times (up to 30 s shown). Exposure to Pi for up to 1 min (not shown) did not cause any further reduction in Ca^{2+} release from the SR (mean

area after Pi exposure for 1 min was 103 ± 27% (n=4; 0CP) and 104 ± 14 (n=4; 10CP) of bracketing controls). Importantly, the effect of Pi on slow twitch-fibers was significantly less at both 20 s and 30 s exposures than for FT fibers with an approximately 2-fold greater effect of Pi being observed in FT fibers.

Phosphate equilibration under paraffin oil.

Because the SR leaks Ca^{2+} when fibers are equilibrated in the presence of EGTA (1 mM), the SR Ca^{2+} content must rapidly decline from the initial level set by the designated load period. In particular, this means that the SR Ca^{2+} content in ST fibers may be greatly reduced after the 30 s equilibration period and this may underlie the reduced effect of Pi on the response to caffeine seen in these fibers. Given that one may expect that the probability of Ca^{2+}-Pi precipitation should then decrease with decreasing SR luminal [Ca^{2+}]. To test this, we created a closed system around the fiber using paraffin oil. Under these conditions, we found that the SR of both fast- and ST fibers did not loose Ca^{2+} over the 30 s equilibration period (<10% in every fiber examined as determined by the change in the area of the force response to Caffeine compared to the pre-equilibration control). Therefore, after a 10 s dip in the 50 mM Pi/ 0 CP solution (1 mM EGTA), fibers were immersed in paraffin oil for a further 20 s (30 s equilibration in total). The area of the response to caffeine was still reduced significantly more in FT compared with ST fibers ($P<0.001$, one-way ANOVA; see Table 2). Importantly, these data confirm that our original observations in FT fibers (33) were not simply an artefact of net losses or gains in SR Ca^{2+}.

<Insert Table 2 here>

Effect of Pi on ST muscle with different SR Ca^{2+} contents.

To determine if the starting SR Ca^{2+} content influenced the effect of Pi in ST fibers we loaded ST fibers for 20 s, 30 s and 2 min. respectively. From Figure 1, a 20 s load equates to approximately 50% of the maximum SR Ca^{2+} content. Consistent SR Ca^{2+} contents at lower load times were difficult to obtain due to the rapid loading rate of ST muscle. Thus, we only examined 20 s and 30 s load times in ST fibers. This is still approximately up to 1.5 fold the endogenous proportion found in FT fibers (see figure 1; see also Trinh & Lamb (37)), but nevertheless, should give some indication if starting SR Ca^{2+} content is important.

<Insert Figure 5 here>

Figure 5 shows the mean data of the area of the caffeine-induced force response in both FT and ST muscle equilibrated with 50 mM Pi/0 CP for 30s after a given load period. Again, ST fibers still appear to be substantially less affected by Pi treatment than FT fibers. Moreover, this effect of Pi on Ca^{2+} release appears to decrease with decreasing SR Ca^{2+} content in ST fibers (although this was not significant).

Pi assisted SR Ca^{2+} loading.

The differences in the effect of Pi observed between FT and ST fibers in this study, may reflect some difference in the way Pi accumulates and precipitates with Ca^{2+} in the SR in these fiber types. To test this, we exploited an old trick used for Ca^{2+} loading the SR of SR vesicle preparations; that is by Pi assistance (18, 20) (see Methods). Figure 6 shows the mean data of these experiments. What was striking

was the extent to which FT fibers could exceed their endogenous maximum SR Ca^{2+} content (initially determined at 2 min loading pCa 6.7) by as much as 13-fold in the presence of Pi, while for ST fibers Ca^{2+} loading in the presence of Pi only doubled the endogenous maximum SR Ca^{2+} content. These data indicated that Pi assisted loading was markedly different between the fiber types.

<Insert Figure 6 here>

Discussion

The present results indicate that: (1) myoplasmic phosphate inhibits SR Ca^{2+} release in both FT and ST skeletal muscle but, with an ~ 2 fold greater effect in FT fibers; (2) the effect of Pi in either fiber type is not different when the cytoplasmic [ADP] is kept very low by the presence of 10 mM CP; (3) the difference in the total SR Ca^{2+} contents between FT and ST fibers can not account for the greater effects of Pi on SR Ca^{2+} release observed in FT fibers; and (4) Pi-assisted Ca^{2+}-loading of the SR results in a substantially increased SR Ca^{2+} content, well above the maximum endogenous content, for FT but, not for ST fibers. These results show that ST fibers are more resistant to the effects of Pi on Ca^{2+} release and that this is possibly due to a reduced ability for the SR to accumulate Ca^{2+}-Pi precipitate.

Effects of Pi on SR Ca^{2+} release in FT- and ST muscle

We have previously shown (33) and confirm here that Pi reduces Ca^{2+} release from the SR in FT fibers consistent with the formation of a Ca^{2+}-Pi precipitate. We also describe a reduced effect of Pi on Ca^{2+} release in ST fibers. Furthermore, we show that the effect of Pi in both FT and ST fibers was not altered when the free cytoplasmic [ADP] was buffered to very low levels by the presence of CP (consistent with previous work by Dutka et al. (11)).

The difference in the effect of Pi between these two fiber types may be attributed to the known differences in the total endogenous SR Ca^{2+} contents (11 mM and 21 mM for FT and ST fibers respectively, normalized to SR volume (16)). It is well known that the ability of Pi to form a precipitate with Ca^{2+} is a function of both free [Ca^{2+}] and [Pi]. The solubility product of Ca^{2+}-Pi has been estimated to be around 5-6 mM^2

(8, 15). Intuitively, one would first expect that the formation of a Ca^{2+}-Pi precipitate would occur at much lower [Pi] and more rapidly in ST fibers given the higher total endogenous SR Ca^{2+} content. However, it is apparent, that even when correcting for Ca^{2+} content differences (see Figure 5), Ca^{2+} release in ST fibers remained less susceptible to the effects of Pi. If anything, as the Ca^{2+} content in ST fibers approached that of FT fibers in this study, the effect of Pi continued to decrease (as one would predict; see Figure 5). Thus, it appears that the different SR Ca^{2+} contents could not simply account for the reduced effect of Pi seen in ST fibers observed here.

Another possibility that may explain the different efficacy of Pi on Ca^{2+} release between FT and ST fibers, is that Pi is less permeable to the SR of ST fibers. We have previously suggested (33) that one possible pathway for Pi entry into the SR in FT fibers is the passive influx into the SR through a non-specific anion channel channel. Laver et al. (23) recently identified this channel as possibly being the small Cl^- channel. Fryer et al, (17) also showed that there may be alternative pathways for Pi entry through an ATP-dependent Pi-transporter. However, at present there has been no previous examination of the presence, density or function of either small Cl- channels or Pi pumps in ST fibers, so we do not know anything about putative role of these channels and pumps in ST fibers. Nevertheless, its seems unlikely that the differences in the effects of Pi on Ca^{2+} release in FT and ST fibers reported here are due to differences in the relative permeability of the SR to Pi; Pi still prevented some Ca^{2+} release in ST, all be it to a smaller extent than FT fibers, indicating that the SR of ST fibers must be reasonably permeable to Pi. Furthermore, oxalate, which is functionally similar to Pi, was also found to be equally permeable to the SR of both FT and ST fibers from human muscle (36).

In FT fibers, 8 mins of Pi-assisted Ca^{2+} loading increased the SR Ca^{2+} content by as much as 13 times above the endogenous maximum Ca^{2+}-content, indicating a substantial capacity of the SR to store Ca^{2+}-Pi (Figure 6). These results are similar to observations of Fryer et al. (17) except that the increased Ca^{2+} content achieved under our conditions was substantially higher than the 3-fold increase reported by Fryer et al. (17). This difference is likely simply due to the different free Ca^{2+} between our loading conditions and theirs (pCa 6.7 vs 7.15). Interestingly, in ST fibers, this Pi-assisted Ca^{2+} loading only doubled the Ca^{2+} content. The reduced capacity to load Ca^{2+} in the presence of Pi in ST fibers may be due to differences in the final equilibrium state between the amount of Pi and Ca^{2+} uptake (and loss) from the SR and Ca^{2+}-Pi precipitation in the SR lumen compared to FT fibers. This could be a consequence (in part) to the presence of different isoforms of calsequestrin in FT and ST SR. Furthermore, the relatively smaller SR volume of rat ST compared to FT fibers may place a simple constraint on the amount of Ca^{2+}-Pi precipitate that can accumulate. Interestingly, this may not be the case for human skeletal muscle, where maximum SR Ca^{2+} storage capacity appears to be similar for both fiber types (36). Nevertheless, this striking difference between FT and ST fibers observed here suggests that Ca^{2+}-Pi precipitation is reduced in ST fibers.

Physiological relevance.

FT and ST fibers perform different roles in the body. Typically, FT fibers are found in muscle groups that are required to provide both rapid contractions and large force outputs while ST fibers are predominantly associated with muscles that perform long duration activity (such as breathing and posture). It is evident then, that these two

major fiber types would have different sensitivities to the many metabolites thought to be involved in fatigue and thus, differences in fatigueability.

ST fibers already have a reduced rate of Ca^{2+} release, force production and relaxation rate (3, 5). As mentioned above, much of this pattern of activity can be explained by the differences in the density and-or function of the DHPRs, RyRs, SR Ca^{2+}-ATPase's (10, 13, 39) as well as the contractile apparatus (35). We show in this study that elevated myoplasmic Pi has a markedly reduced effect on SR Ca^{2+}-handling in ST fibers compared to FT fibers. This reduced (but not absent) effect of Pi in ST fibers may have an important functional role in controlling the activity of ST fibers during normal activity and also prevent the development of fatigue. It is interesting that the SR of ST fibers is essentially full (15). This would be expected to restrict Ca^{2+} uptake, as the SR Ca^{2+}-ATPases would be prevented from pumping Ca^{2+} across such a large gradient. It is known that the dynamic range of myoplasmic Pi between rest and activity is smaller in ST than in FT fibers (~6-30 mM in ST and ~1-50 mM in FT fibers). A reduced sensitivity to Pi in ST fibers (for reasons described above) would have two major effects. The first is that the effective reduction in the free [Ca^{2+}] in the SR (when some Pi enters the SR and precipitates with Ca^{2+}) would allow Ca^{2+}-uptake, which would otherwise be impossible if the SR was completely full. The second is that the seemingly reduced capacity to accumulate Ca^{2+}-Pi precipitate in the SR of ST fibers would minimise any Pi induced reduction in the free Ca^{2+} available for release. We know in FT fibers that the amount of Ca^{2+} released per action potential is relatively fixed and not affected until the free Ca^{2+} in the SR is substantially reduced (< 200μM; 35) and this is true even when the SR Ca^{2+} content is well above the normal endogenous content. In ST fibers, perhaps the initial extra

reserve of Ca^{2+} ensures that even with some Pi entry in to the SR, the free Ca^{2+} available for release remains relatively unaffected. Thus, some reduction in SR Ca^{2+} in ST fibers would still allow sufficient Ca^{2+} release to initiate contraction. In FT fibers however, the greater dynamic range of Pi coupled to a smaller total SR Ca^{2+} content, greatly decreases the amount of free releasable Ca^{2+} sufficiently to reduce force output and contribute to fatigue.

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Figure 1. Relationship between load time and the area of the caffeine-induced force response in FT an ST fibers.

Both FT (n=3) and ST (n=4) fibers were loaded with Ca^{2+} (Load solution, pCa 6.7 - see Methods) for differing times until the SR was saturated with Ca^{2+}. The area of the caffeine-induced force response after each load period was normalized to the maximum load time (120 s). Areas were corrected using methods described by Macdonald and Stephenson (26). Curves were fitted to the data using a single exponential (parameters: max = 100 and min = 0).

Figure 2. Effect of 50 mM Pi in a single skinned FT skeletal muscle fiber in the presence and absence of 10 mM creatine phosphate. From left to right and top to bottom, the trace shows the data obtained in a single FT fiber subjected to the load-release protocol described in the methods. Following a 30 s Ca^{2+} load (to approximately match the typical endogenous FT total SR Ca^{2+} content (see Figure 1), the fiber was equilibrated for 30 s in either a standard K-HDTA solution, a 50 mM Pi/ 0 CP solution or a 50 mM Pi/ 10 CP solution, each containing 1 mM total EGTA. The subsequent force responses to the Caffeine solution (indicated by solid bars below force trace; slow time scale of 1s) are indicative of the total releasable Ca^{2+} content after the appropriate equilibration period (e.g. Control and post Pi treatment). Each exposure to the Caffeine solution fully empties the SR of Ca^{2+} such that subsequent Ca^{2+} loads result in similar total SR Ca^{2+} contents. Equilibration for 30 s in a Pi solution in either the absence or presence of CP resulted in a similarly large reduction in the force response to Caffeine solution (compare responses 2 to 5) which indicated that the loaded SR Ca^{2+} was not easily released, as previously described (30). Responses were normalized to the average control force response before and

after Pi treatment. Maximum Ca^{2+}-actived force in pCa 4.5 (Max) and strontium (pSr 5.4) exposure are shown at the faster time scale of 0.5 sec.

Figure 3. Effect of 50 mM Pi exposure on FT and ST fibers in the absence and presence of creatine phosphate. Mean area of the caffeine-induced force responses (± SEM, *n* in brackets) for *A*: 50 mM Pi/ 0 CP treatments and *B*: 50 mM Pi/10 CP treatments. Mean data compared with a two-way ANOVA; P value in figure indicates overall significance between fiber types. Bonferroni post-test analysis: * $P<0.05$; ** $P<0.01$. Y-axis: Area is expressed as the percentage of the bracketing control responses before and after Pi treatment (see Figures 1 and 2). X-axis: Exposure time to appropriate Pi solution.

Figure 4. Effect of 50 mM Pi in a single skinned ST skeletal muscle fiber in the presence and absence of creatine phosphate (CP).

From left to right and top to bottom, the trace shows the data obtained in a single ST fiber subjected to the load-release protocol described in the methods. Following a 2 min Ca^{2+} load (to fully load the SR), the fiber was equilibrated for 30 s in either a standard K-HDTA solution, a 50 mM Pi/ 0 CP solution or a 50 mM Pi/ 10 CP solution, each containing 1 mM total EGTA. The subsequent force responses to the Caffeine solution (indicated by solid bars below force trace) are indicative of the total releasable Ca^{2+} content after the appropriate equilibration period (e.g. Control and post Pi treatment). Each exposure to the Caffeine solution fully empties the SR of Ca^{2+} such that subsequent Ca^{2+} loads result in similar total SR Ca^{2+} contents. It can be seen that exposure to Pi in either the presence or absence of CP had little affect on the subsequent force response to the Caffeine solution (compare 2^{nd} and 5^{th} responses);

compare with the larger affect of Pi treatment observed in a single FT fiber in Figure 2. Responses were normalized to the average control force response before and after Pi treatment. Maximum Ca^{2+}-actived force in pCa 4.5 (Max) and strontium (pSr 5.4) exposure.

Figure 5. Effect of Pi on caffeine-induced calcium release in ST fibers at different SR Ca^{2+} contents. ST fibers were loaded at different times (2 min (n=13), 30 s (n=5), and 20s (n =3)) to vary the SR Ca^{2+} content and then exposed to a 50 Pi/0CP solution for 30 s (see methods). The mean relative area ± SE of the caffeine-induced force response as a percentage of bracketing control responses is shown. It can be seen that the area of the response to caffeine for FT fibers (n = 7) was significantly smaller compared to all other treatments for ST fibers ($P<0.01$ for each comparison using a one-way ANOVA).

Figure 6. Pi-assisted SR Ca^{2+} loading in skinned FT and ST fibers.
The SR of both FT and ST fibers were initially exposed to the Load solution (see Table 1) for 2 min to maximally load the SR with Ca^{2+} (refer to Figure 1). The area of the force response to caffeine subsequently obtained after a 2 min load was the response with which all subsequent responses to caffeine following Pi-assisted loading were normalized to. The graph shows the normalized area of the caffeine-induced force response after fibers were exposed to a 50 mM Pi/10 mM CP solution with 1 mM total EGTA, pCa 6.7 (e.g. modified Load solution) for different times. P value in figure indicates overall significance using a Two-Way ANOVA (Bonferroni Post Hoc: *, $P<0.05$).

Table 1. Composition of skinned fiber solutions.

Solution	Hepes (mM)	HDTA (mM)	$EGTA_t$ (mM)	ATP (mM)	CP (mM)	Mg^{2+}_f (mM)	Ca^{2+} (μM)	P_i (mM)
K-HDTA	90	50	0.025	8	10	1	0.1	—
Load*	90	50	1	8	10	1	0.2	—
50Pi/0CP*	100	11.5	1	8	—	1	<0.01	50
50Pi/10CP*	90	11.5	1	8	10	1	<0.01	50
Max activating	90	—	50	8	10	1	20	—
High relaxing	90	—	50	8	10	1	<0.001	—
Caffeine	90	50	1.0	8	10	0.02	—	—
Wash*	90	50	0.5	8	10	1	<0.01	—
pSr 5.4	90	—	50	8	10	1	<0.001	—

All solutions contained (mM): K+, 126; Na+, 36; NaN3, 1; pH 7.10 ± 0.01 at room temperature (23 ± 2°C). Total Ca^{2+} and Mg^{2+} were varied appropriately to give the indicated free concentration. pSr (-log (Sr^{2+}]) 5.4 solution contained 4μM free Sr^{2+}. The Caffeine solution contains 30 mM caffeine. Subscripts t and f define total and free, respectively. The asteric (*) indicates solutions derived from the K-HDTA solution. CP, creatine phosphate; HDTA, hexamethylene-diamine-tetraacetic acid; P_i, inorganic phosphate (derived from KH_2PO_4).

Table 2: Effect of 50mM Pi exposure on the peak caffeine-induced force response in fast-and slow-twitch fibers equilibrated in the presence and absence of paraffin oil.

Peak	*Slow-twitch*		*Fast-twitch*	
	0CP	10CP	0CP	10CP
30 s exposure (no oil)	75±6 (13)	71±4 (11)	41±5 (7)*	45±6 (5)*
30 s (oil)	82±3 (4)	NA	44±4 (3)*	NA

Data is mean ± SE (*n* in brackets) of the caffeine-induced force response after either a 30 s equilibration in 50 mM Pi with or without CP (10 and 0 respectively) and no subsequent equilibration in oil (no oil) or after a 10 s treatment in 50 mM Pi 0CP followed by immersion in oil for a further 20 s (30 s total time). The superscript (*) indicates a significant difference ($P < 0.001$) from the corresponding ST value using a one-way ANOVA. NA indicates not applicable.

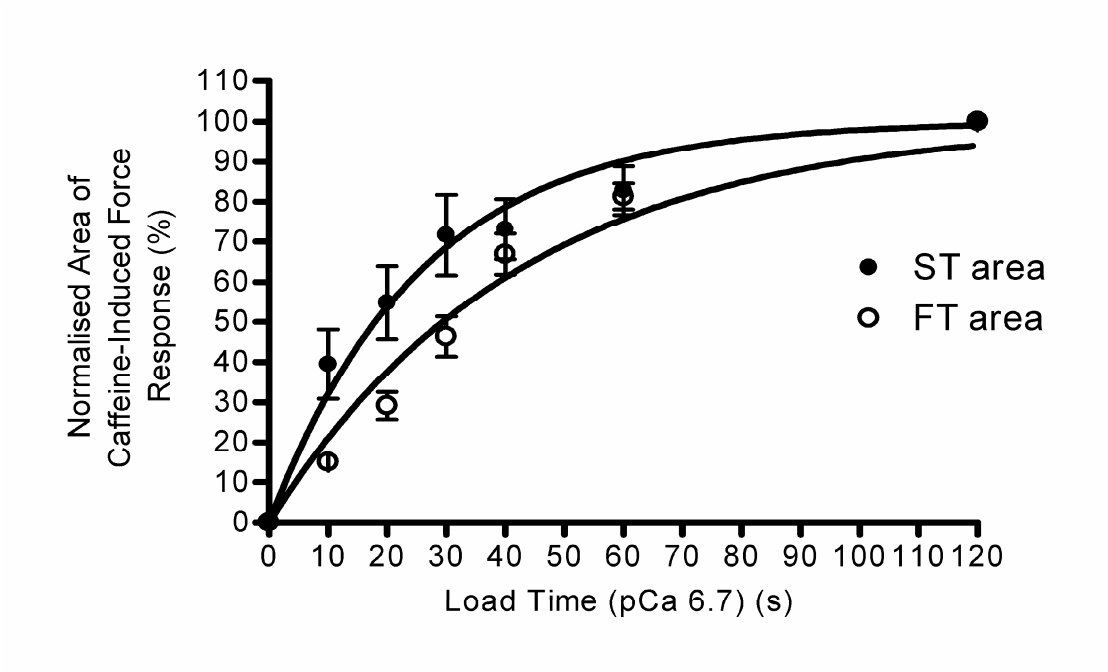

Normalised Area of Caffeine-Induced Force Response (%)
110
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Load Time (pCa 6.7) (s)
ST area
FT area

Figure 1

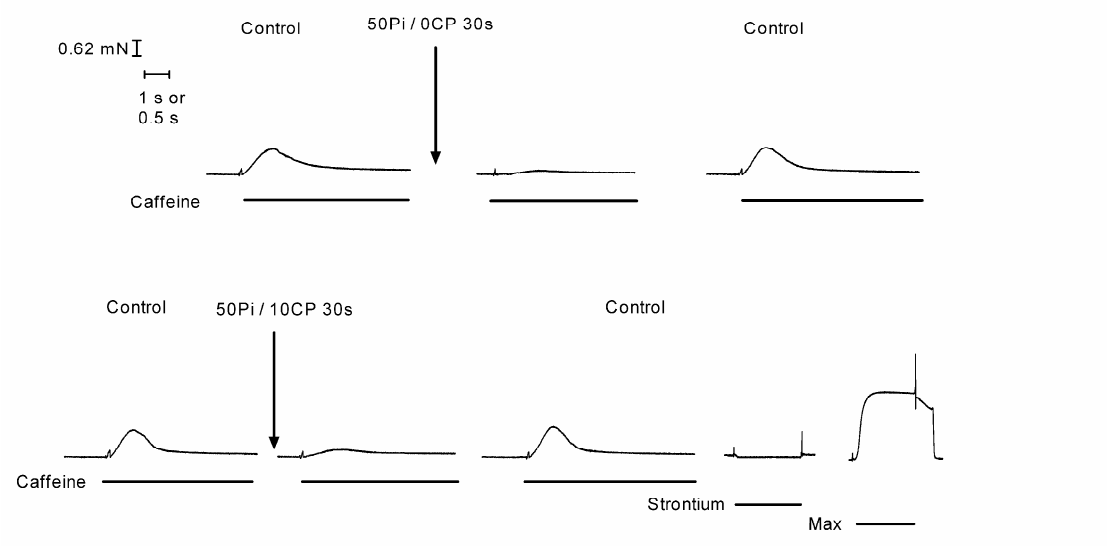

Control
50Pi / 0CP 30s
Control
0.62 mN
1 s or
0.5 s
Caffeine
Control
50Pi / 10CP 30s
Control
Caffeine
Strontium
Max

Figure 2

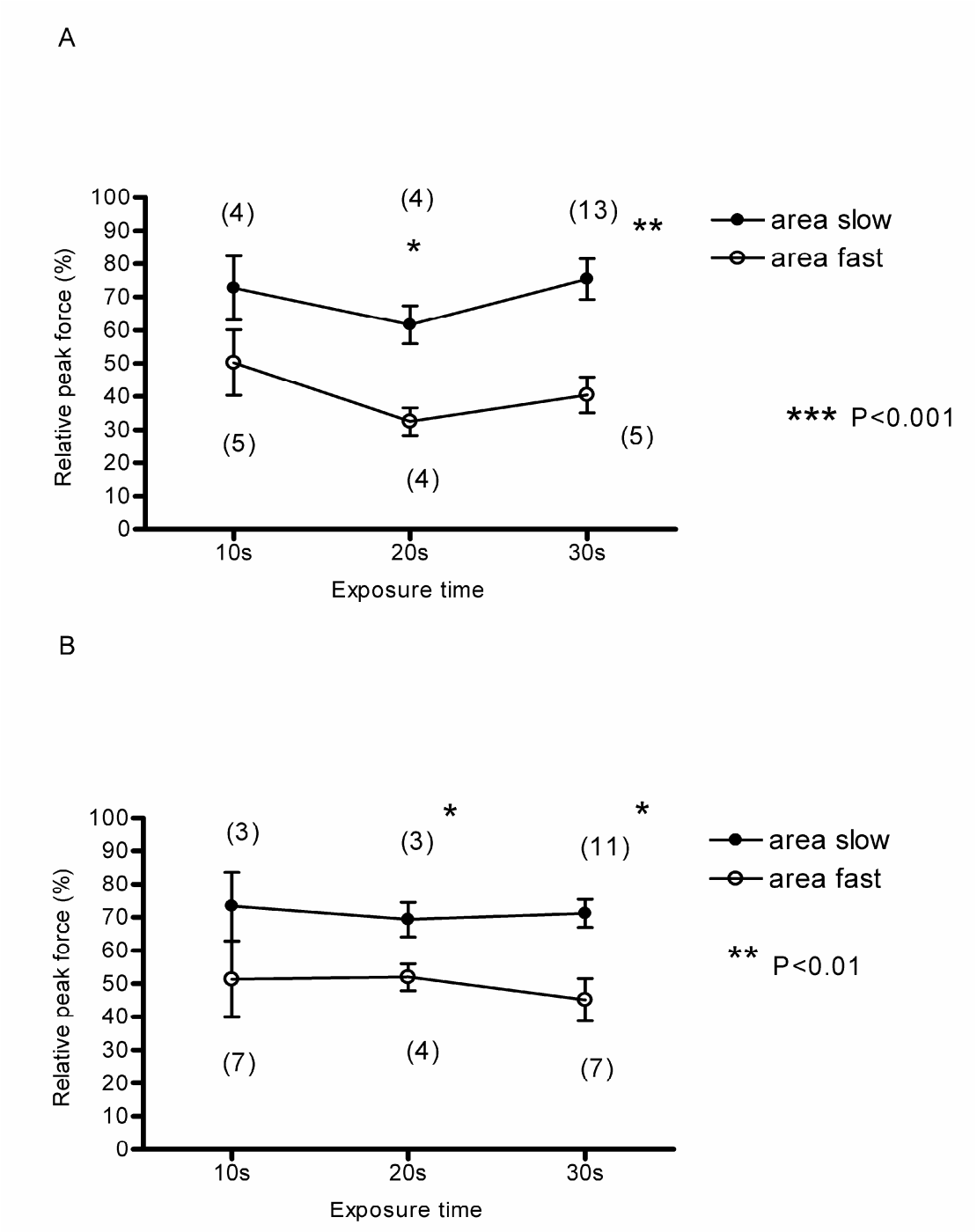

A
Relative peak force (%)
100
90
80
70
60
50
40
30
20
10
0
(4)
(4)
(13)
**
*
(5)
(5)
(4)
10s
20s
30s
Exposure time
area slow
area fast
*** P<0.001
B
Relative peak force (%)
*
*
(3)
(3)
(11)
(7)
(4)
(7)
10s
20s
30s
Exposure time
area slow
area fast
** P<0.01

Figure 3

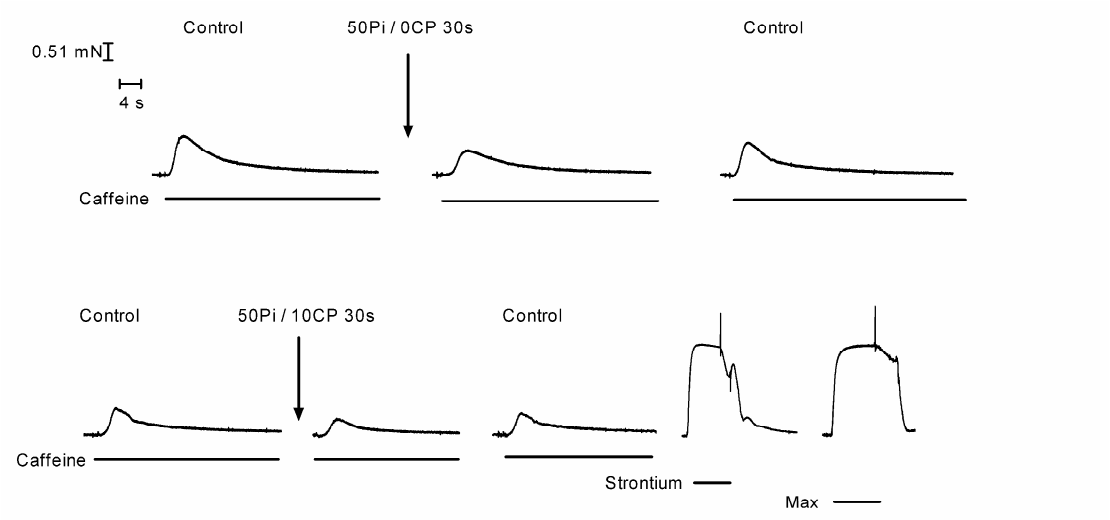

Control
50Pi / 0CP 30s
Control
0.51 mN
4 s
Caffeine
Control
50Pi / 10CP 30s
Control
Caffeine
Strontium
Max

Figure 4

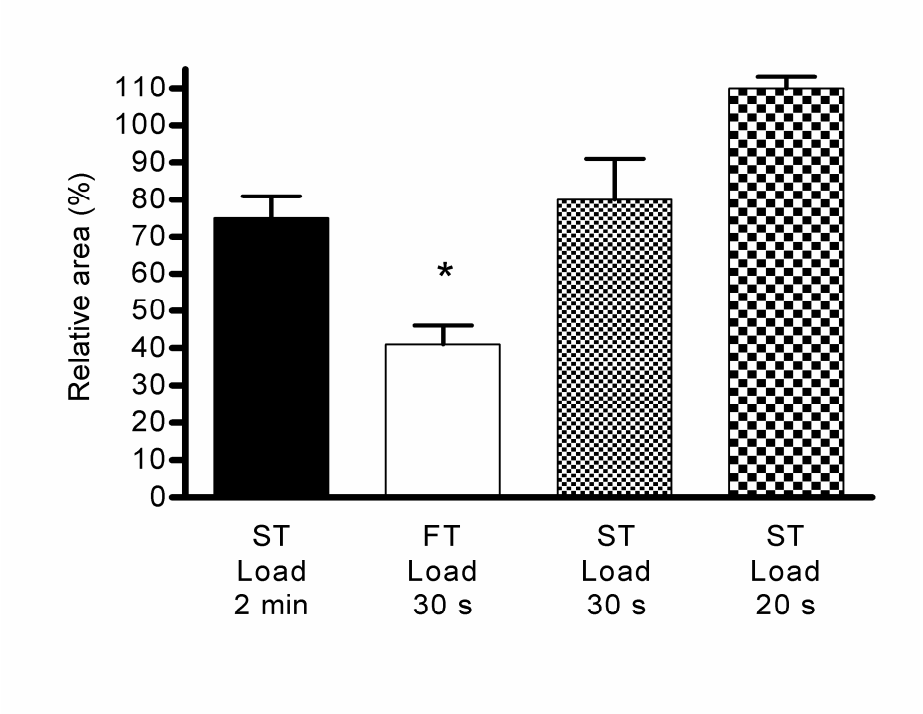

Relative area (%)
110
100
90
80
70
60
50
40
30
20
10
0
*
ST
Load
2 min
FT
Load
30 s
ST
Load
30 s
ST
Load
20 s

Figure 5

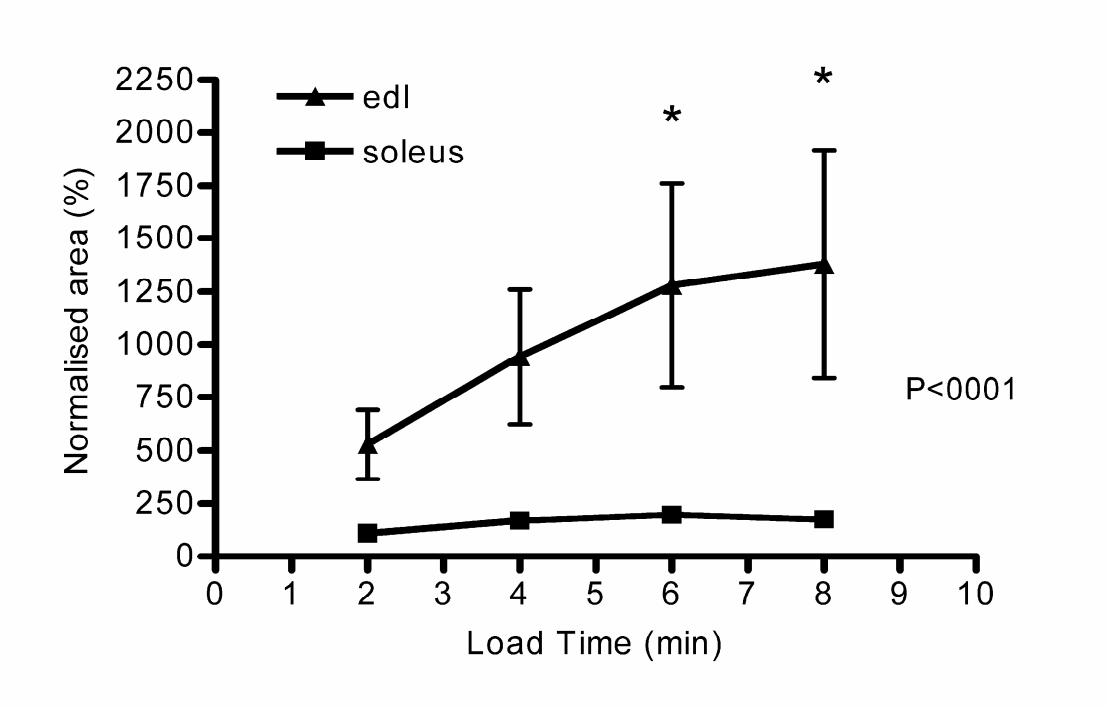

2250
2000
1750
1500
1250
1000
750
500
250
0
Normalised area (%)
edl
soleus
*
*
P<0001
0
1
2
3
4
5
6
7
8
9
10
Load Time (min)

Figure 6