Effect of physiological levels of caffeine on Ca\(^{2+}\) handling and fatigue development in \textit{Xenopus} isolated single myofibers

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Rosser JI, Walsh B, Hogan MC. Effect of physiological levels of caffeine on Ca\(^{2+}\) handling and fatigue development in \textit{Xenopus} isolated single myofibers. \textit{Am J Physiol Regul Integr Comp Physiol} 296: R1512–R1517, 2009. First published March 4, 2009; doi:10.1152/ajpregu.90901.2008.—The purpose of the present study was to determine whether exposure to exogenous physiological concentrations of caffeine influence contractility, Ca\(^{2+}\) handling, and fatigue development in isolated single \textit{Xenopus laevis} skeletal muscle fibers. After isolation, two identical contractile periods (separated by 60-min rest) were conducted in each single myofiber (n = 8) at \(20^\circ\)C. During the first contractile period, four fibers were perfused with a noncaffeinated Ringer solution, while the other four fibers were perfused with a caffeinated (70 \(\mu\)M) Ringer solution. The order was reversed for the second contractile period. The single myofibers were stimulated during each contractile period at increasing frequencies (0.16, 0.20, 0.25, 0.33, 0.50, and 1.0 tetanic contractions/s), with each stimulation frequency lasting 2 min until fatigue ensued, defined in this study as a fall in tension development to 66% of maximum. Tension development and free cytosolic [Ca\(^{2+}\)] (fura-2 fluorescence spectroscopy) were simultaneously measured. There was no significant difference in the peak force generation, time to fatigue, cytosolic Ca\(^{2+}\) levels, or relaxation times between the noncaffeinated and caffeinated trials. These results demonstrate that physiological levels of caffeine have no significant effect on \textit{Xenopus} single myofiber contractility, Ca\(^{2+}\) handling, and fatigue development, and suggest that any ergogenic effects of physiological levels of caffeine on muscle performance during contractions of moderate to high intensity are likely related to factors extraneous to the muscle fiber.

Numerous studies have demonstrated that caffeine ingestion results in a distinct improvement in endurance activities (6, 15, 16, 17, 36). It has been suggested (see Ref. 43) that caffeine-induced improvements in exercise performance are facilitated via increased free fatty acid utilization (7, 40), central and peripheral nervous system activation (9), and by a direct effect on skeletal muscle (33). There is some evidence (32, 44) that the direct effect of caffeine on skeletal muscle in improving endurance performance may be due to an increase in force output for a given neural input. Furthermore, some studies also indicate that caffeine may assist in short-term power activity (5, 42, 48), while other studies do not support this conclusion (4, 8, 18, 19, 20).

Most studies that have investigated the effects of caffeine on muscle performance have been performed using human subjects, in which it is difficult to deduce the specific effects of caffeine, or in isolated muscle preparations using caffeine concentrations that are toxic to humans (2, 10, 22, 38). Studies using intact, isolated single muscle fibers (1, 2) have shown that high concentrations of caffeine (5 mM) can increase Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), increase tension development in single tetanic contractions, and interestingly, almost completely reestablish maximum tension development in fatigued single myofibers by restoring calcium release from the SR to nonfatigued levels (21, 47). Figure 1 illustrates this latter effect in a representative single fiber that has been stimulated to fatigue (i.e., severe contraction-induced reduction in tension development); demonstrating that acute and rapid exposure to a supraphysiological concentration (5 mM) of caffeine to the solution surrounding the fatigued myofiber causes an immediate increase in Ca\(^{2+}\) release from the SR and thereby restores force development to prefatigue levels. Similar findings have been demonstrated in studies that use isolated muscle fibers to delineate mechanisms of fatigue in skeletal muscle (2, 45, 47), and it has been suggested (32) that this effect of caffeine on intracellular Ca\(^{2+}\) handling may be one possible mechanism for the beneficial effect of caffeine on muscle performance. Although it is clear that high levels of caffeine can strongly influence muscle function and contractility, it remains less certain what effect physiological levels of caffeine have on intracellular responses in working muscle (see Ref. 21).

The objective of the current study was to examine the effects of a physiological concentration of caffeine (70 \(\mu\)M) on contractility, calcium handling, and fatigue in isolated single skeletal muscle fibers. Many of the confounding variables present in whole muscle and human studies are easily controlled in the isolated single-fiber preparation. In particular, neurological considerations are removed so that intracellular properties of myofibers can be investigated independently from the well-known effects of caffeine on the nervous system. Given that caffeine ingestion improves human endurance performance, and that large doses of caffeine can dramatically restore contractility in fatigued single fibers (see Fig. 1), we tested the hypothesis that physiological concentrations of caffeine would improve contractile performance in single \textit{Xenopus} skeletal muscle fibers through alterations in intracellular Ca\(^{2+}\) handling.

**METHODS**

\textit{Experimental preparation.} Adult female \textit{Xenopus laevis} were doubly pithed and decapitated. Lumbrical muscles II-IV were removed, and single living muscle fibers were microdissected from the muscle. Dissections and experiments were performed in Ringer solution (112 mM NaCl, 1.87 mM KCl, 0.82 mM CaCl\(_2\), 2.38 mM NaHCO\(_3\), 0.07 mM NaH\(_2\)PO\(_4\), 1.0 mM EGTA) at \(20^\circ\)C and 7.0 pH. All procedures were approved by the University of California-San Diego Animal Care and Use Committee and conform to National Institutes of Health standards.
lengths of 340 nm and 380 nm, and the resulting fluorescence emissions at 510 nm were divided (340 nm/380 nm) to obtain the Ca$^{2+}$-dependent signal, as we have done previously (28, 41). Fluorescence was measured with a Photon Technology International illumination and detection system (DeltaScan model), integrated with a Nikon inverted microscope with a 40× Fluor objective.

**Measurements.** All waveform analyses were performed using AcqKnowledgeIII software. Tension development during each contraction was compared with the highest peak tension within that contractile run and reported as relative tension. Relative [Ca$^{2+}$]c measurements were standardized in a similar fashion. Five individual 340/380 nm excitation ratios (peak [Ca$^{2+}$]c) were averaged at each measurement time point and compared with the average of the five highest ratios within that run. Relative resting [Ca$^{2+}$]c measurements (340/380 nm baseline) were averaged in a similar fashion and compared with the lowest resting levels within that run.

**Statistics.** Two-way repeated-measures ANOVA was performed for the statistical analysis. Results are reported as means ± SE. In all analyses, the 0.05 level of significance was used.

**RESULTS**

Figure 2 illustrates a typical fatigue run in which force and relative free cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]c) were simultaneously measured for a single muscle fiber bathed in both caffeine and noncaffeinated solutions. Multiple aspects of contractile performance were analyzed, including peak tension, time to fatigue (defined as 66% of peak tension), and relaxation times at peak and fatigue time points. The Ca$^{2+}$ ratio (a higher ratio corresponding to higher levels of cytosolic Ca$^{2+}$) and the change in the baseline of the calcium throughout a fatigue run were measured; five adjacent contractions were averaged for all of the calcium calculations.

**Peak tension.** A comparison of the peak tension generated during caffeine and noncaffeinated runs revealed no significant difference between the two runs. The average peak tension generated for caffeine and noncaffeinated runs was 0.88 ± 0.55 mV and 0.84 ± 0.45 mV, respectively. There was no significant difference in peak tension development between caffeine and noncaffeinated treatments within the same fiber.

**Muscle fatigue.** Similarly, there was no significant variation in time to fatigue between caffeine and noncaffeinated conditions. The average times to fatigue in caffeine and noncaffeinated runs were 566 ± 125 and 548 ± 120 s, respectively (Fig. 3). There was wide variation among different fibers, but relative consistency between trials involving the same fiber.

**Relaxation time.** The elapsed time between the end time point of peak tension development for a contraction and the point at which the developed tension returned to 33% of its baseline was used as a measure of relaxation time. The average relaxation times during the contraction inducing peak tension of caffeine and noncaffeinated runs were 0.12 ± 0.05 and 0.11 ± 0.4 s, respectively. At the fatigue time point, caffeine and noncaffeinated relaxation times of 0.33 ± 0.15 and 0.27 ± 0.08 s were not significantly different from each other; however, these relaxation times were significantly greater than the relaxation times at the peak contractile time points (Fig. 4).

**Calcium.** The calcium ratios, which provide relative indexes of [Ca$^{2+}$]c, during contractions at the peak tension time point at the beginning of the contractile work bout were not significantly different between caffeine and noncaffeinated runs.
In addition, the ratios during the contracted state at the point of fatigue were identical with respective averages of 1.16 ± 0.51 and 1.16 ± 0.50 (Fig. 5), but this fatigue [Ca^{2+}]_c was significantly less than the [Ca^{2+}]_c measured at the peak tension time point. Baseline cytosolic Ca^{2+} (i.e., the [Ca^{2+}]_c during the time the fiber is in the relaxed state between contractions) was increased significantly at the fatigue time point relative to the baseline cytosolic Ca^{2+} at the beginning of the work bout in both the caffeinated (53 ± 0.14%) and noncaffeinated runs (47 ± 0.18%; not significantly different between the caffeinated vs. noncaffeinated treatments). This change in Ca^{2+} handling during the relaxation time between consecutive contractions can be seen clearly in the Fig. 2, top, with cytosolic Ca^{2+} baseline levels clearly not being restored to prefatigue levels as fatigue ensued. Finally, there was no significant difference in the force/Ca^{2+} ratio (an index of myofilament sensitivity to Ca^{2+}) between the caffeinated (0.53 ± 0.06) and noncaffeinated (0.51 ± 0.05) runs at any time point, and this ratio did not significantly change from the peak tension time point to the fatigue time point.

**DISCUSSION**

The results of this present study, using a Xenopus isolated single skeletal muscle fiber model, showed no significant excitation/contraction effects of exposure to a physiological extracellular concentration of caffeine (70 μM) on contractile characteristics, time to fatigue, or calcium handling during a fatiguing contractile period.

*Caffeine and muscle function.* Human studies have repeatedly demonstrated caffeine’s ergogenic effects on athletic performance. Several studies have demonstrated an increase in muscle endurance (increased time to fatigue) in athletes given a dose of caffeine prior to exercise compared with both control and placebo groups (6, 7, 17, 36, 48). However, the effect of caffeine on muscle contractility and function during moderate-to-high intensity exercise remains less clear (17, 36, 43). While some studies reveal little or no significant improvement in high-intensity performance with the ingestion of caffeine (4, 19, 20), other studies have shown an increase in this variable.

**Fig. 2.** A: Cytosolic [Ca^{2+}] (top) and force development (bottom) in a representative single muscle fiber bathed in 70 μM caffeine Ringer solution during tetanic contractions of increasing stimulation frequency leading to fatigue. B: [Ca^{2+}], and force in the same fiber after a 60-min rest period while bathed in noncaffeinated Ringer solution during the fatigue run.

**Fig. 3.** Mean time until the fatigue time point (defined as the point at which force generated had decreased to 66% of the initial maximum force) during the fatigue runs. There was no statistically significant difference in time to fatigue between caffeinated and noncaffeinated trials.

**Fig. 4.** Mean relaxation time (elapsed time between the end time point of the maximum force of a contraction and the point at which the force returned to 33% of its baseline) of contractions at the peak tension and at the point of fatigue in caffeinated and control trials. Relaxation times were significantly slower at the point of fatigue (significant difference *P < 0.001) compared with the initial maximum force, but there was no statistically significant difference in relaxation times between caffeinated and noncaffeinated treatments at the two time points.
Thus, it has been suggested that caffeine may enhance muscle contractility by increasing the release of calcium ions, decreasing reuptake of calcium ions, or increasing myofibril sensitivity to calcium ions (21, 32, 44).

To test the direct effect of caffeine on muscle function, several studies have used isolated and electrically stimulated whole muscle under varying conditions. It has been demonstrated that a large dose of caffeine, directly applied to isolated whole muscle, has the effect of increasing muscle tension as a result of increasing calcium release from the SR (14, 26, 38), although this dramatic effect of high levels of caffeine on severely fatigued blood-perfused whole muscle is not universal (22). However, these studies were performed using millimolar concentrations of caffeine, well above the level that would be toxic to humans (17, 27). Rather, the plasma caffeine concentration of caffeine in humans is usually between 20 and 50 micromolar with an upper limit of 70 micromolar (17). Furthermore, it has been demonstrated in whole muscle that decreasing the concentration of caffeine results in a drastic reduction in the ability of caffeine to rescue contractile function in fatigued muscle (35). Thus, although the results from studies using supraphysiological concentrations of caffeine do offer some insights about the potential effects that caffeine can have on muscles and, in particular, shed light on the mechanisms of muscle fatigue, these studies do not adequately describe the effects of caffeine in an exercising human.

There have been very few other studies examining the effects of physiological levels of caffeine on isolated muscle function. In one study (24), the application of 70 μM caffeine did not significantly affect force, work, or power in whole mouse muscles subjected to work loops. However, a separate study by the same laboratory suggested a small increase (2–3%) in peak force and work but no changes in the pattern of fatigue or recovery after caffeine exposure (25). Our present study used physiological levels of caffeine (70 μM) on isolated single muscle fibers to more closely investigate the cellular responses of physiological caffeine application. This model allowed muscle fibers to be subjected to a standard fatigue run of increasing contraction frequency, similar to human exercise. Furthermore, the present study measured both tension and calcium handling simultaneously to examine the proposed mechanisms of caffeine directly on myofibers, independently of neurological considerations. While the results of the present study demonstrated great variability in tension generation and calcium handling between fibers, the single fiber model allows each fiber to be used as its own control. The results of the current study demonstrated that caffeine, at physiological levels (70 μM), had no significant effect on peak tension or time to fatigue. Although there was a significant difference in relaxation time between peak tension development and at the point of fatigue, there was no significant difference in the magnitude of that change between caffeine and noncaffeinated runs.

Thus, these results demonstrate that exposure to physiological concentrations of caffeine does not significantly alter peak tension development, endurance (time to fatigue), and the pattern of fatigue in these single fibers. It should be noted, however, that although the Ca²⁺ handling and response to caffeine in single intact fibers are similar between mammalian and amphibian fibers, it remains unknown as to whether in mammalian fibers at 37°C, there may be some direct cellular
effect at the caffeine concentrations used in this study, that was not seen at room temperature in these amphibian fibers.

Caffeine and calcium handling. One of the mechanisms proposed for caffeine’s effects on muscle contractility has been increased calcium release by the SR (see Ref. 21). It has been well established that increased calcium release results in greater generation of muscle tension (1, 13, 30). There is also evidence that caffeine increases the number and duration of open SR calcium channels (30, 29). Thus, it is reasonable to conclude that caffeine increases force by increasing calcium release. However, no studies, excluding the present study, have simultaneously measured force generation and cytosolic \([Ca^{2+}]\) in single fibers exposed to physiological levels of caffeine. The results of this current study showed the characteristic decrease in peak \([Ca^{2+}]\) as fatigue developed throughout each contractile bout (see Fig. 5). However, there was no significant difference in peak or baseline \([Ca^{2+}]\) between caffeinated and noncaffeinated trials, again indicating that exposure to physiological concentrations of caffeine did not alter \(Ca^{2+}\)-handling properties and subsequent contractile performance in these Xenopus myofibers at 20°C.

With the onset of fatigue in the present study, the baseline level of cytosolic calcium steadily increased as calcium accumulated in the cytosol faster than it could be pumped back into the SR, resulting in slowing of the myofilament relaxation time (see Fig. 2). However, the increase in baseline \([Ca^{2+}]\) in the fatigued state, even though substantial, was not sufficient to impede total relaxation of force generation to resting levels between stimulation time points (see Fig. 2). Allen and Westerblad (2) indicated that 5 mM caffeine can slow the calcium pumps, and thereby slow the reuptake of calcium, thus increasing the baseline level of calcium more drastically in caffeinated surroundings. In the present study, the caffeine treatment did not have a significantly different effect on baseline calcium at either the start and end of fatigue runs compared with the noncaffeinated treatment, indicating that low micromolar concentrations of caffeine do not cause a substantial change in either calcium release or calcium pump activity. A caffeine-related increase in calcium release, combined with slower calcium pumping, would cause an accumulation of calcium in the cytosol, which has been shown to decrease calcium release (46). It is possible that this negative feedback stabilizes the calcium levels when caffeine is administered in low doses (<70 \(\mu\)M) but can be overwhelmed with large doses of caffeine (millimolar concentrations). Finally, there is generally a strong correlation between calcium release and force generation (force/\(Ca^{2+}\) ratio) that is indicative of the myofilament sensitivity to \(Ca^{2+}\) (45). In the present study, we found no difference in the force/\(Ca^{2+}\) ratio between the caffeine and noncaffeine conditions at either the peak time point or the fatigue time point, indicating that this level of caffeine did not influence myofilament sensitivity to \(Ca^{2+}\).

Perspectives and Significance

In conclusion, the results of the present study demonstrated no effect of exposure to a physiological concentration of caffeine (70 \(\mu\)M) on fatigue, contractility, and calcium handling in isolated Xenopus single myofibers at 20°C. These results suggest that any ergogenic effects of physiological levels of caffeine on muscle performance during contractions of moderate to high intensity are likely related to factors extraneous to the muscle fiber itself. However, even though the bioenergetic response to caffeine of amphibian and mammalian muscle is similar, extrapolation of these present results to the in vivo human exercise response needs further examination.

GRANTS

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REFERENCES


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