Intracellular calcium accumulation following eccentric contractions in rat skeletal muscle in vivo: role of stretch-activated channels

Takashi Sonobe,1 Tadakatsu Inagaki,1 David C. Poole,2,3 and Yutaka Kano1

1Departments of Applied Physics and Chemistry, University of Electro-Communications, Chofu, Tokyo, Japan; 2Departments of Anatomy, Physiology and Kinesiology, Kansas State University, Manhattan, Kansas; and 3School of Sports and Health Sciences, University of Exeter, Exeter, Devon, United Kingdom

Submitted 9 November 2007; accepted in final form 9 January 2008

Sonobe T, Inagaki T, Poole DC, Kano Y. Intracellular calcium accumulation following eccentric contractions in rat skeletal muscle in vivo: role of stretch-activated channels. Am J Physiol Regul Integr Comp Physiol 294: R1329–R1337, 2008.—Although the accumulation of intracellular calcium ions ([Ca2+]i) is associated with muscle damage, little is known regarding the temporal profile of muscle [Ca2+]i, under in vivo conditions, and, specifically, the effects of different contraction types [e.g., isometric (ISO); eccentric (ECC)] on [Ca2+]i. For 90 min at rest, an in vivo vs. in vitro preparation would better maintain initial [Ca2+]i. 2) Compared with ISO, ECC contractions (50 contractions, 10 sets, 5-min interval) would lead to a greater increase of [Ca2+]i. 3) Elevated [Ca2+]i during ECC would be reduced or prevented by the stretch-activated ion channel blockers streptomycin and gadolinium (Gd3+). Spinotrapezius muscles of Wistar rats were exteriorized (in vivo) or excised (in vitro). [Ca2+]i was evaluated by loading the muscle with fura 2-AM using fluorescence imaging. [Ca2+]i rose progressively beyond 40 min at rest under in vitro but not in vivo conditions during the 90-min protocol. In vivo [Ca2+]i increased more rapidly during ECC (first set) than ISO (fifth set) (P < 0.05 vs. precontraction values). The peak level of [Ca2+]i was increased by 21.5% (ISO) and 42.8% (ECC) after 10 sets (both P < 0.01). Streptomycin and Gd3+ abolished the majority of [Ca2+]i increase during ECC (69 and 86% reduction, respectively; P < 0.01 from peak [Ca2+]i, of ECC). In conclusion, in vivo quantitative analyses demonstrated that ECC contractions elevate [Ca2+]i, significantly more than ISO contractions and that stretch-activated channels may play a permissive role in this response.

spino trap ezius; muscle damage; streptomycin; gadolinium

THE CONTRACTION-RELAXATION CYCLE OF MYOCITIES IS REGULATED BY CHANGES OF INTRACELLULAR Ca2+ CONCENTRATION ([Ca2+]i). RESTING MYOCITIES MAINTAIN [Ca2+]i UNDER ~0.1 μMOL (2). WHEN MYOCITIES CONTRACT, THERE IS A TRANSIENT ELEVATION OF [Ca2+]i THAT HELPS TRIGGER MYOCYTE CONTRACTION. IN NONFATIGUED AND NONDAMAGED MYOCITIES, THE ASSEMBLY DETECTION AND RECOVERY AFTER CONTRACTION, [Ca2+]i DECREASES IMMEDIATELY. However, long repeated contractions induce myocyte fatigue (reduction of tetanic force), myocyte damage, and prolonged elevation of [Ca2+]i after contraction(s) (15, 43).

It is well known that eccentric (ECC) contractions induce muscle damage (8, 26, 44), and it has been suggested that one principal cause of that damage is high [Ca2+]i (1, 17). Moreover, if ECC does cause increased [Ca2+]i, it is possible that stretched-activated ion channels (SAC) involved in this response (1, 54–56) and the associated muscle damage. Franco and Lansman (11, 12) initially reported SAC function in skeletal muscle and noted that the channels were blocked by streptomycin and gadolinium (Gd3+). Recently, Yeung and colleagues (54–56) demonstrated that SAC blocker treatment prevented an increase in the resting [Ca2+]i in isolated single fibers from the mdx mouse, a model of human Duchenne muscular dystrophy, following ECC contractions. Furthermore, it has been suggested that inhibition of SAC during ECC contraction attenuates activation of muscle growth-related signaling pathways (42). These phenomena may be associated with perturbations of the intracellular ionic environment; for example, [Ca2+]i levels may rise through ECC contraction-induced SAC activation.

Because of the difficulty in measuring [Ca2+]i, under in vivo conditions, almost all investigations of [Ca2+]i have been performed under in vitro conditions. However, such in vitro conditions are likely to perturb [Ca2+]i, regulation, and, therefore, development of an in vivo preparation, capable of resolving [Ca2+]i, in single fibers, might provide a unique and valuable opportunity to better understand the role of [Ca2+]i, in muscle function and dysfunction. Bioimaging techniques can visualize intracellular ions directly (9, 39, 50, 51), and, whereas measurements of [Ca2+]i are potentially feasible under in vivo conditions, studies have been too thick for microscopy, and the recent studies of [Ca2+]i, have used isolated or cultured single myocytes. Unfortunately, such isolated or cultured cells have quite a different environment than in vivo skeletal muscle with respect to their absence of a microcirculation, different oxygen and substrate availabilities, and metabolism, among other considerations (43, 47).

Since the development of the spinotrapezius intravital microscopy preparation by Gray in 1973 (18), this muscle has served as a keystone for the understanding of muscle microvascular control. The spinotrapezius is sufficiently thin to permit transmission light microscopy, is composed of all three major mammalian muscle fiber types (10), and has an oxidative capacity similar to that of the human quadriceps (30). To date, there are a few reports of [Ca2+]i measured using bioimaging techniques in the spinotrapezius muscle (23, 48), but the effects of repeated isometric (ISO) and ECC contractions on [Ca2+]i have not been investigated.

The purpose of the present investigation was to test the following original hypotheses in the spinotrapezius muscle of...
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healthy rats. 1) Compared with the surgically excised in vitro spinotrapezius, the in vivo preparation (i.e., exteriorized, as for intravital microscopy; Refs. 18, 38, 45, 46) prolonged (90 min) observation at rest would not elevate [Ca2+]. 2) An extended series of ECC would elevate [Ca2+], to a greater extent than ISO contractions. 3) The elevated [Ca2+], accompanying ECC contractions would be prevented or substantially reduced by the SAC blockers.

METHODS

Animals

Male Wistar rats (n = 36, Japan SLC), 8–12 wk of age, were used in this study. Rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. All experiments were conducted under the guidelines established by the Physiological Society of Japan and were approved by University of Electro-Communications Institutional Animal Care and Use Committee. The rats were anesthetized with intraperitoneal injection of pentobarbital sodium (70 mg/kg ip), and supplemental doses of anesthesia were administered, as needed.

In Vivo Muscle Preparation

The spinotrapezius was exteriorized, as described previously (3, 25, 27, 28, 38). Briefly, the right spinotrapezius muscle was carefully accessed through an ∼4-cm-long midline incision through the skin, starting at the lower cervical level and extending caudally to the upper lumbar vertebral level. Exteriorization was performed with as little disruption as possible to minimize tissue damage. With the exception of the distal feed artery, all of the vascular and nervous connections remained intact as the caudal perimeter of the muscle was dissected free of connective tissue attachments. The exposed spinotrapezius muscle was attached to a thin-wire horsehoe around the caudal periphery by five to six equidistant sutures placed around the caudal perimeter. For the contraction protocols, electrodes were placed on the dorsal spinotrapezius surface proximal to the motor point and along the caudal periphery, facilitating indirect whole muscle contractions. The muscle surface was kept moist by superfusing with warmed Krebs-Henseleit buffer solution (KHB; 132 NaCl, 4.7 KCl, 21.8 NaHCO3, 2 MgSO4, 2 CaCl2 mM), equilibrated with 95% N2–5% CO2 and adjusted to pH 7.4, at 37°C. All other drugs were dissolved into KHB solution. In the experiments examining the effects of SAC blockade, streptomycin and Gd3+ were used. Aqueous stock solutions of streptomycin and GdCl3 were diluted in normal KHB to final concentrations of 200 and 20 µM, respectively (52, 55, 56). Fluorescence Ca2+ indicator fura 2-AM (5 mM, Dojindo Laboratories) was dissolved in dimethyl sulfoxide and Pluronic F-127 (final < 0.1%) and dispersed into the KHB solution at a final concentration of 20 µM. Muscles were incubated in fura 2-AM/KHB solution for 30 min on a 37°C hotplate to facilitate AM esterase activity. After incubation, muscles were rinsed with dye-free KHB solution to remove nonloaded fura 2-AM, and the microscopy protocol was initiated within 15 min.

In Vitro Muscle Preparation

The preparation was similar to the in vivo condition, except that the rostral (scapular) perimeter of the muscle was also dissected free from connective tissue connections. Subsequently, the detached muscle was fixed in an immovable clamp at its rostral extremity, while the caudal boundary was sutured to the horseshoe, as described for the in vivo preparation above.

Microscopy and Fluorescence Measurement

The fura 2-loaded spinotrapezius muscles were mounted on the 37°C glass hotplate (Kitazato Supply), which reduced any movements due to breathing and cardiac contractions, and observed by fluorescence microscopy using a ×10 objective lens (Nikon). After ensuring that the muscle was not grossly damaged and supported blood flow, a sampling area (∼880 × 663 µm) was selected using branching vessels from the main feed artery as landmarks, and bright-field images were captured (Fig. 1). Thereafter, 340-nm and 380-nm wavelength excitation light was delivered using a xenon lamp equipped with appropriate fluorescent filters, and pairs of fluorescence images were captured for ratiometry at 1.6 pixels/µm monitor resolution.

Image Analysis

When the spinotrapezius muscles were observed in bright-field illumination, muscle fibers and microvessels, including capillaries, were clearly visualized. Whereas there was no arteriolar or capillary red blood cell (RBC) flow in the in vitro preparation, these microvessels maintained good blood flow until the end of the measurement period in the in vivo preparation. In any preparations in which the arteriolar blood flow ceased during the observation period, the results from that muscle were not analyzed. After selecting an appropriate region of interest, the spinotrapezius was observed fluorescently, and 340- and 380-nm excitation wavelength fluorescent filters were switched manually. Images were converted to 340/380 ratio image by ImageJ software (National Institutes of Health), and the 340/380 ratio image data were averaged over the whole area sampled. The 340/380 images were assumed to indicate [Ca2+]i (Fig. 2).

Images were captured by a high-sensitivity charge-coupled device digital camera (DP70, Olympus) using image-capture software (OP Control, Olympus). From captured bright-field images, mean sarcomere length was determined from sets of five consecutive in-register sarcomeres (i.e., distance between 6 consecutive A-bands). This distance was measured to within ±0.1 µm, and that distance was divided by 5 to yield sarcomere length. This procedure was performed five times within the image, where sarcomeres were visible, to obtain a mean sarcomere length for each viewing field. Fluorescence images were captured at 5-s exposure, and this protocol was maintained during all experiments. Before the experiment, background fluorescence intensity was checked in non-fura 2-loaded spinotrapezius muscles. Captured images were analyzed using ImageJ. The 340/380-nm ratio image was calculated using the “divide mode” from the 340- and 380-nm excitation images to the 32-bit gray-scale image. The fluorescence intensity of serial ratio images was normalized to the starting point of each experiment. [Ca2+]i measurements were performed.

Fig. 1. Bright-field microscopy image of rat spinotrapezius muscle in vivo. Image area was selected using first or second branching vessels emanating from main feeding artery as the visual landmark. #, Arterioles; *, venule. As detailed in METHODS, almost all vessels (feed artery, arterioles, capillaries, venules) maintained blood flow throughout the procedures. Scale bar = 100 µm.
formed within specific regions of interest selected from the whole muscle image, which included multiple muscle fibers. Using this technique, we evaluated the behavior of \([\text{Ca}^{2+}]_i\) within individual muscle fibers in whole in vivo and in vitro muscle.

**Experimental Protocols**

Animals were divided into six groups: 1) in vitro control (n = 5); 2) in vivo control (n = 8); 3) in vivo ISO contractions (n = 7); 4) in vivo ECC contractions (n = 7); 5) in vivo ECC contractions with streptomycin (n = 5); and 6) in vivo ECC contractions with Gd\(^{3+}\) (n = 4). Groups 1 and 2 addressed hypothesis 1 by comparing in vitro with in vivo muscle. Groups 3 and 4 addressed hypothesis 2 by comparing \([\text{Ca}^{2+}]_i\), changes across different contraction types (i.e., ISO and ECC). Finally, groups 5 and 6 addressed hypothesis 3 by investigating the involvement of SAC in \([\text{Ca}^{2+}]_i\), accumulation.

**Group 1: In vitro control group.** The resting (nonstimulated) control experiment was performed in vitro by surgically isolating and loading the spinotrapezius muscle with fura 2 before microscopy. Sequential fluorescence images were captured every 5 min for 90 min. Each pair of 340- and 380-nm images was analyzed and quantified. Quantified data were graphed as changes from precontraction (baseline) levels.

**Group 2: In vivo control group.** The protocol was the same as for group 1, except that the muscle was exteriorized with the principal vascular and neural pathways maintained intact.

**Group 3: In vivo ISO contractions group.** The time course of \([\text{Ca}^{2+}]_i\), change was observed after each of 10 discrete sets of ISO muscle stimulation, in a similar fashion to that described previously (46). Specifically, each set consisted of the muscle being stimulated tetanically at resting spinotrapezius sarcomere length (100 Hz, 5–8 V, 10.220.33.6 on May 28, 2017 http://ajpregu.physiology.org/ Downloaded from
stimulus duration 700 ms, 2.6- to 2.8-μm sarcomere length) every 3 s for 2.5 min (i.e., 50 contractions). Pairs of fluorescence images were captured precontraction and after each set of contractions, as well as at the end of the 5-min between-set recovery (immediately before initiation of the subsequent set of contractions).

**Group 4: In vivo ECC contractions group.** Muscle lengthening ECC contractions in in vivo spinotrapezius muscle were compared with ISO contractions in group 3. The ECC contraction protocol was modified slightly from that reported previously (26). The motor device coupled with electro-stimulator (model RU-72, NEC Medical Systems) evoked strain via the caudal edge of the muscle, which was attached to the wire horseshoe and delivered a stretch (lengthening) of 10% of resting sarcomere length (i.e., to ~3.0 μm). Muscle lengthening was started 0.2 s after initiation of electrical stimulation and immediately returned to resting length at the end of electrical stimulation. Other settings and measurements were the same as for protocol 3 above (ISO contractions).

**Groups 5 and 6: Streptomycin and Gd³⁺ groups.** Protocol was the same as for group 4 above, except that the KHB superfusate contained either streptomycin or Gd³⁺.

**Force Measurement**

The wire horseshoe attached to the spinotrapezius muscle was connected by fine wire to a strain gauge. Torque (0–10 mN•m, scale) was monitored by computer using Mac Lab/8s (A/D Instruments Pty.) via strain-gauge-linked motor device during all contraction protocols. The first and last five contractions of sets 1, 5, and 10 were averaged and plotted graphically as the index of fatigue.

**Statistical Analysis**

All statistical analyses were performed in Prism version 4.0 (GraphPad Software). A two-way repeated-measures ANOVA and Bonferroni post hoc test was used for in vitro vs. in vivo, ISO vs. ECC, and ECC vs. streptomycin or Gd³⁺ comparisons. A one-way repeated-measures ANOVA and Bonferroni post hoc test was used for relative force comparison. Measured values are presented as means ± SE. Significance was established at P < 0.05.

**RESULTS**

The time course of [Ca²⁺]i changes during 90-min resting conditions is depicted in Fig. 3. Under the in vitro conditions, the 340/380-nm ratio value increased systematically beyond 50 min. Specifically, a significant change (P < 0.05) was observed at 50 min after starting the protocol and reached a peak of 47.8 ± 1.2% above baseline at the 90-min point. In contrast, there was no significant change over the 90-min observation period for the in vivo muscles.

These results justified selection of the in vivo spinotrapezius preparation for all five subsequent contraction protocols. The fixed muscle length ISO contraction condition demonstrated a moderate degree of [Ca²⁺]i accumulation, which became significantly elevated above baseline only after five sets of contractions (Fig. 4). Values after the final contraction set (no. 10) were elevated 21.5 ± 5.7% above precontraction baseline. In contrast, the ECC protocol induced a significant [Ca²⁺]i increase of 10.9 ± 3.1% (P < 0.01) after the first set of contractions, and this systematically increased to 42.8 ± 5.3% above baseline after the final set of contractions. Thus this rate of [Ca²⁺]i change over 10 sets of ECC contractions was two times greater than that for the ISO group. There were significant differences between ISO and ECC contractions after seven sets.

The SAC blockers streptomycin and Gd³⁺ significantly reduced the increase of [Ca²⁺]i during ECC contractions (Fig. 5). In fact, the [Ca²⁺]i profile in the presence of streptomycin and Gd³⁺ resembled closely that seen for ISO contractions (Fig. 4), with [Ca²⁺]i not increasing above precontraction baseline. This effect was most dramatic after the 10th set of ECC contractions, when the [Ca²⁺]i accumulation was reduced from 42.8 ± 5.3 to 13.4 ± 2.1 and 5.9 ± 2.7% above precontraction baseline by streptomycin and Gd³⁺, respectively. Moreover, the extensive numbers of high [Ca²⁺]i fibers that appeared in ECC contractions without SAC blockers were almost completely absent at the end of the streptomycin and Gd³⁺ protocols. This observation was supported by the bright-field observation, where the majority of fibers retained their normal non-hypercontraction state.

Figure 6 shows the changes of relative tetanic force, which was measured during all contraction protocols in spinotrapezius muscle. ISO force decreased ~20–30% within a set, and at the final 10th set it was significantly decreased to 41.9 ± 7.5% of prefatigue conditions. While ECC also indicates decreasing tetanic force within a set, there was not any significant attenuation of eccentric force production throughout 10 sets of contractions. SAC blockers streptomycin and Gd³⁺ with ECC contractions did not affect eccentric force.

The foregoing data showed the calculated mean values over the whole imaged tissue area (880 × 663 μm). However, we noted that the elevation of muscle fiber [Ca²⁺]i did not present uniformly across all fibers. Rather, some single fibers or a few localized fibers evidenced an increased [Ca²⁺]i, in both in vitro and, to a lesser extent, in vivo (contractions) protocols. Figure 7 demonstrates two distinctly different profiles of [Ca²⁺]i increase during the in vitro protocol. Specifically, most fibers demonstrated a gradual increase of [Ca²⁺]i uniformly along the fiber length. However, the starred fiber [Ca²⁺]i shows a discrete Ca²⁺ "front" that propagates over time further along the fiber. This propagation of Ca²⁺ was also observed in in vivo contraction protocols. Most of the fibers that evidenced a high [Ca²⁺]i under fluorescence analysis elicited excessive sarcomeric contraction in bright field.

![Fig. 3. The time course of [Ca²⁺]i changes during 90-min resting conditions. Changes of [Ca²⁺]i are depicted as ratio values relative to initial level in resting rat spinotrapezius muscles in vitro and in vivo conditions. Values are means ± SE (in vitro: n = 5, in vivo: n = 8). Significance compared with initial level for each condition: *P < 0.05, **P < 0.01. Significant difference between in vitro and in vivo conditions for the same time points: #P < 0.05, ###P < 0.01.](http://ajpregu.physiology.org/)

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Fig. 4. Effect of 10 sets of isometric (ISO) and eccentric (ECC) contractions in spinotrapezius muscles in vivo on [Ca\textsuperscript{2+}]. Fluorescence intensity was measured at precontraction, postcontraction, and after rest period between contraction periods. Values shown are means ± SE (ISO: n = 7, ECC: n = 7). Significance compared with precontraction level for each condition: *P < 0.05, **P < 0.01. Significant difference compared with ISO and ECC condition for the same time points: #P < 0.05, ##P < 0.01.

DISCUSSION

The present investigation demonstrates the viability of an in vivo spinotrapezius preparation to explore the effects of different types of muscle contractions on [Ca\textsuperscript{2+}], accumulation. The principal original findings include the following. 1) ISO contractions were associated with a modest elevation of [Ca\textsuperscript{2+}], that was manifested only after five contraction bouts. In marked contrast, ECC contractions caused an immediate and substantial elevation of [Ca\textsuperscript{2+}]. 2) The SAC blockers, streptomycin and Gd\textsuperscript{3+}, abolished or significantly reduced [Ca\textsuperscript{2+}] accumulation, suggesting that SAC are responsible, in large part, for the ECC contractions-induced [Ca\textsuperscript{2+}], elevation in healthy muscle.

Methodological Considerations

The spinotrapezius muscle has formed a cornerstone of our understanding of muscle microcirculatory control (18, 21, 24, 29, 34). As well as possessing optical properties requisite for intravitral microscopy, it is composed of all three major muscle fiber types (10) and has an oxidative capacity similar to that found in the human quadriceps (30). As detailed in METHODS, the spinotrapezius can be exteriorized for microscopy while preserving vascular and neural pathways, and sarcomere length can be set with precision, if required (38). These considerations are paramount if in vivo microcirculatory structure and function and, therefore, muscle viability are to be maintained. In the present investigation, it was crucial that each muscle fiber could be discerned clearly by bright-field microscopy, and it was ensured that arterioles and capillaries sustained RBC flow throughout the experimental period.

For the present investigation, we specifically chose fura 2 because, using a ratiometry technique, this indicator facilitates the determination of [Ca\textsuperscript{2+}], without problems associated with variations of tissue and liquid thicknesses, amount of intracellular indicator, and/or the specific power of the luminous source (51). We set the image exposure time to 5 s, which facilitated capture of the fluorescence images required. Comparison was made between fura 2-loaded and unloaded muscles, and it was determined that there was no significant background fluorescence at 340- and 380-nm wavelengths. In addition, because fura 2 quenching Mn\textsuperscript{2+} solution (19) decreased fluorescence intensity, it was believed that the fluorescence signal in this study could be attributed exclusively to fura 2 binding with [Ca\textsuperscript{2+}]i.

In Vitro vs. In Vivo

Historically, it has been common practice to superfuse in vitro muscle preparations with very high (nonphysiological) partial pressures of O\textsubscript{2} (P\textsubscript{O2}) between 100 and 600 Torr. Specifically, microvascular P\textsubscript{O2} is ~30 Torr in the resting in vivo spinotrapezius muscle (5). Whereas, in the absence of an intact microcirculation, P\textsubscript{O2} values of 100–600 Torr are necessary to provide O\textsubscript{2} for mitochondrial oxidative phosphorylation, high P\textsubscript{O2} values, in and of themselves, can be injurious.
to the tissue. Hence, the in vitro preparation is far from ideal in terms of hyperoxic/hypoxic damage, as well as provision of substrates such as free fatty acids and glucose. In the present investigation, we chose to compare the in vivo preparation to an in vitro one that was not damaged by nonphysiologically high Po2, recognizing that it would become overtly hypoxic as resting metabolism utilized the small available O2 reserves in RBCs, dissolved in plasma and extra- and intracellular fluids and on myoglobin. Consequently, the [Ca2+]i elevation observed herein likely resulted from the process of hypoxia/anoxia-induced muscle cell degradation (33, 36, 49). This phenomenon was also reported by Terada et al. (47), who measured [Ca2+]i in the thin (similar to the spinotrapezius) epitrochlearis muscle. Despite provision of a glucose and O2-supplemented buffer, [Ca2+]i was significantly elevated after 70 min of observation. Collectively, these results indicate that survivability of muscle tissue depends strongly on the maintenance of resting blood flow. Indeed, under such in vitro conditions, whole muscle tissue may be more damaged than single-muscle fibers (57). In contrast, in the present investigation, there were no significant changes in resting muscle [Ca2+]i under the circulation-intact in vivo conditions. Our microscopic observation area in the present investigation included at least the main feed artery and/or the first- and second-order branching arteriolar vessels (1A and 2A). It was ensured that, for the in vivo condition, these vessels maintained blood flow during the experiment. We consider that this is the most likely explanation for the muscle fibers in the in vivo (in contrast to the in vitro) preparation maintaining cellular function, such that [Ca2+]i,
accumulation was prevented and [Ca\(^{2+}\)], therefore, remained at resting levels (Fig. 3).

**ISO vs. ECC Contractions**

Although some reports indicate that [Ca\(^{2+}\)], accumulation may not occur following shorter bouts of ISO contractions (4, 22), longer lasting bouts of muscle contractions that cause fatigue do elicit muscle [Ca\(^{2+}\)], accumulation (7, 15, 16). It is possible that, initially, during ISO contractions, Ca\(^{2+}\) originates exclusively from the sarcoplasmic reticulum (SR; i.e., intracellularly) with quantitatively little Ca\(^{2+}\) migrating across the sarcolemma. However, more chronic stimulation protocols, such as the 10 bouts used herein, may cause a gradual [Ca\(^{2+}\)] accumulation that becomes evident after five sets of contractions and increased progressively thereafter. In agreement with McBride et al. (32), who have shown no SAC activation with concentric contractions, we speculate that the most likely source of the elevated [Ca\(^{2+}\)], was SR-released Ca\(^{2+}\), and its accumulation reflects a progressive inability for the SR to recover during each resting period.

On the other hand, [Ca\(^{2+}\)], elevation of ECC contracted muscle was larger and faster compared with the ISO condition. As shown in many previous studies (1, 2, 4), our results suggest that ECC contraction induces high [Ca\(^{2+}\)] accumulation. There are two major potential sources of this Ca\(^{2+}\); either SR (i.e., intracellular), or the extracellular space where [Ca\(^{2+}\)] is \(\geq 10,000\)-fold greater than resting [Ca\(^{2+}\)]. It has been recognized that ECC contraction can damage the sarcolemmal microstructure, and that SAC are present in the muscle cell membrane (12). In consideration of the above, we sought to block the sarcolemmal SAC to test the novel hypothesis that these channels were responsible for the [Ca\(^{2+}\)] accumulation caused by ECC contractions.

**Effect of SAC Blocker**

It has been determined that streptomycin and Gd\(^{3+}\) function as SAC blockers in working skeletal muscle from healthy rat (31, 32, 42, 53) or mdx mouse (52, 55, 56). However, the specificity of these agents is often brought into question. While it has been suggested that aminoglycosides and Gd\(^{3+}\) can block the sarcolemmal SAC to test the novel hypothesis that these channels were responsible for the [Ca\(^{2+}\)] accumulation caused by ECC contractions.

**Tetanic Force and Contraction Protocols**

It has been established that voluntary concentric contractions induced a greater loss of force than voluntary ECC contractions (37). Similarly, ECC contractions have a greater resistance to fatigue than ISO and concentric contractions compared at the same electrically stimulated contraction intensity (6). We found that, while repeated ECC contractions tend to decrease tetanic force, there was not a significant attenuation of force production, at least over the number of contractions evaluated herein (Fig. 6). Also, because no difference in fatigability occurs following ECC, with or without blockers, we presume that tetanic force during repeated ECC contractions was not dependent on resting Ca\(^{2+}\) level.

**Ca\(^{2+}\) Propagation in Muscle Fibers**

One advantage of using bio-imaging is the ability to observe [Ca\(^{2+}\)], changes in real time by image analysis for each muscle fiber in whole tissue. To evaluate the time course of [Ca\(^{2+}\)], changes, digitizing was performed on ratio images that included 10–20 muscle fibers. This digitizing area (880 \(\times\) 663 \(\mu\)m, \(-0.6 \text{mm}\)) was considerably larger than a previous study in single fibers (50 \(\times\) 50 \(\mu\)m; Ref. 56) and in vivo skeletal muscle (100 \(\times\) 100 \(\mu\)m; Ref. 48). In the latter study, [Ca\(^{2+}\)], changes were averaged over multiple muscle fibers. Using ratio-image analysis, a longitudinal progression of [Ca\(^{2+}\)], accumulation resembling a wave could be identified in single muscle fibers from the equated-digitized graph. These [Ca\(^{2+}\)], accumulations were quite different from the acute [Ca\(^{2+}\)], transients associated directly with each contraction. We speculated that the source of this Ca\(^{2+}\) influx from an effective increase of sarcolemml permeability rather than SR damage and the absence of such behavior under the SAC inhibiting conditions.

Heterogeneous [Ca\(^{2+}\)], accumulation patterns among fibers were observed under our experimental conditions. Because the tetanic contractions were elicited from direct supramaximal electrical stimulation, this heterogeneity of [Ca\(^{2+}\)], could not be explained by selective motor unit recruitment during contraction. It is likely that different muscle fiber types are more susceptible to such damage than others. For example, Ivanics and colleagues (23) have described a selective damage (and [Ca\(^{2+}\)], accumulation) in oxidative type I fibers following an ischemia-reperfusion protocol in the spinotrapezius muscle. In contrast, Suzuki and colleagues (46) found that an ischemia-reperfusion protocol selectively damaged那些 fibers with a low oxidative capacity (most likely type IIB fibers, as visualized using rhodamine-123 to identify relative mitochondrial content). When muscle is injured consequent to ECC contractions, muscle fibers may be selectively damaged, and, as these fibers subsequently produce less force, the remaining healthy fibers may be subjected to greater stresses and are likely to
suffer progressively more injury (14). In the present investigation, those fibers with the greatest \([Ca^{2+}]\) tended to be the larger diameter fibers, consistent with (but not proof of) selective damage to type II fibers.

**Perspectives and Significance**

This investigation has demonstrated that, under in vivo conditions, ECC contractions induce a more rapid and far greater \([Ca^{2+}]\) accumulation than ISO contractions. Moreover, it appears that SAC are mechanistically involved in this ECC contraction-induced \([Ca^{2+}]\) accumulation and, therefore, any damage resulting from the high \([Ca^{2+}]\). Future experiments designed specifically to determine the fiber-type specificity of this effect would prove valuable.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Dr. Tadashi Nakamura and Dr. Hideki Shirakawa for helpful comments on the experiments.

**GRANTS**

This study was supported in part by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (no. 18700526) and the Uehara Memorial Foundation.

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