Changes in [Ca\(^{2+}\)]_i induced by several glucose transport-enhancing stimuli in rat epitrochlearis muscle

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Terada, Shin, Isao Muraoka, and Izumi Tabata. Changes in [Ca\(^{2+}\)]_i, induced by several glucose transport-enhancing stimuli in rat epitrochlearis muscle. J Appl Physiol 94: 1813–1820, 2003. First published January 24, 2003; 10.1152/japplphysiol.00780.2002.—The purpose of the present investigation was to establish a method for estimating intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]_i) in isolated rat epitrochlearis muscles. Epitrochlearis muscles excised from 4-wk-old male Sprague-Dawley rats were loaded with a fluorescent Ca\(^{2+}\) indicator, fura 2-AM, for 60–90 min at 35°C in oxygenated Krebs-Henseleit buffer. After fura 2 loading and subsequent 20-min incubation, the intensities of 500-nm fluorescence, induced by 340- and 380-nm excitation lights (F\(_{\text{total340}}\) and F\(_{\text{total380}}\)), were measured. The fluorescence specific to fura-2 ([F\(_{\text{fura340}}\) and F\(_{\text{fura280}}\)) were calculated by subtracting the non-fura-2-specific component from F\(_{\text{total340}}\) and F\(_{\text{total380}}\), respectively. The ratio of F\(_{\text{fura340}}\) to F\(_{\text{fura280}}\) was calculated as R, and the change in the ratio from the baseline value (ΔR) was used as an index of the change in [Ca\(^{2+}\)]_i. In resting muscle, ΔR was stable for 60 min. Incubation for 20 min with caffeine (3–10 mM) significantly increased ΔR in a concentration-dependent manner. Incubation with hypoxic Krebs-Henseleit buffer for 10–60 min significantly elevated ΔR, depending on the duration of the incubation. Incubation with 50 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide for 20 min significantly elevated ΔR (P < 0.05). No significant increases in ΔR were observed during incubation for 20 min with 2 mM 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside or with 2 mM/ml insulin. These results demonstrated that, by using the fura 2-AM fluorescence method, the changes in [Ca\(^{2+}\)]_i, can be monitored in the rat epitrochlearis muscle and suggest that the method can be utilized to observe quantitative information regarding [Ca\(^{2+}\)]_i that may be involved in contraction- and hypoxia-stimulated glucose transport activity in skeletal muscle.

fura 2; hypoxia; 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside; insulin; caffeine; intracellular Ca\(^{2+}\) concentration

IN MAMMALIAN SKELETAL MUSCLE, intracellular free calcium (Ca\(^{2+}\)) has been assumed to play important roles not only in excitation-contraction coupling (31) but also in signal transduction as a second messenger modulating a variety of essential cellular functions (2, 14).

Generally, the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) is estimated with fluorescent Ca\(^{2+}\) indicators, such as fura 2 and indo 1. Previous investigations using these fluorescent Ca\(^{2+}\) indicators developed methods for measuring [Ca\(^{2+}\)]_i in mammalian skeletal muscle (4, 5, 24, 26, 39, 40) or cultured muscle (15, 30, 42) cells. Although these previous investigations using frog skeletal muscle and cultured skeletal muscle cells provided valuable information on Ca\(^{2+}\) in skeletal muscles, [Ca\(^{2+}\)]_i in intact mammalian muscle must be measured if we are to evaluate the role of Ca\(^{2+}\) concentration in skeletal muscle, because frog skeletal muscle and cultured cells may have different characteristics from mammalian skeletal muscle cells. These differences include the lack of additivity of the maximal effect of insulin and the contraction on glucose transport activity (22), the requirement of protein synthesis for the reversal of enhanced glucose transport activity after contraction (17) in frog sartorius muscle, the absence of sarcoplasmic reticulum (SR) from myoblasts (16), or the low expression levels of the GLUT-4 isoform of the glucose transporter in cultured cells (16). Töth et al. (43) established a method to assess [Ca\(^{2+}\)]_i levels in rat skeletal muscle in vivo. However, no method for measuring [Ca\(^{2+}\)]_i in rat skeletal muscle in vitro has yet been reported. For the purpose of studying the effects of the combination of various physiological and pharmacological stimuli on [Ca\(^{2+}\)]_i, and its involvement in glucose transport activity in rat skeletal muscle, and in vitro method for assessing [Ca\(^{2+}\)]_i in isolated rat muscle is necessary.

Because Ca\(^{2+}\) has been postulated to be involved in enhanced glucose transport stimulated by contraction and/or hypoxia (23), several previous investigations (10, 45, 46) adopted in vitro pharmacological approaches using pharmacological stimulators (caffeine, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7)) or inhibitors (dantrolene, 9-aminoacridine) of

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Ca\textsuperscript{2+} release from SR and evaluated the effects of the postulated changes in Ca\textsuperscript{2+} on glucose transport activity in rat epitrochlearis muscle. The epitrochlearis muscle is a thin muscle only ~20 fibers thick (34). This short diffusion distance makes it possible to maintain adequate ATP and creatine phosphate levels during prolonged incubation in vitro (32). A number of studies of glucose transport have demonstrated the suitability of the epitrochlearis muscle for in vitro incubation experiments (7, 10, 45–47). Therefore, because this muscle in oxygenated incubation medium in vitro is known to maintain its physiological functions similar to those under an in vivo condition for a long time, we thought it would be possible to monitor the changes in [Ca\textsuperscript{2+}]; in rat epitrochlearis muscle in vitro by using a fluorescent Ca\textsuperscript{2+} indicator.

In terms of glucose transport, hypoxia stimulates glucose transport activity in skeletal muscles (7, 10, 46), as do insulin and contraction. Although the mechanism underlying hypoxia-stimulated glucose transport has not yet been determined, one candidate for this mechanism is the increase in [Ca\textsuperscript{2+}]; (7, 10, 46). However, no report has observed the actual involvement of Ca\textsuperscript{2+}; in hypoxia-stimulated glucose transport, because no technique for monitoring the change in [Ca\textsuperscript{2+}]; in the rat epitrochlearis muscle has been established. Such a technique would be a valuable tool for evaluating the role of [Ca\textsuperscript{2+}]; in regulating glucose transport in skeletal muscle.

Therefore, the purposes of the present investigation were 1) to establish a method for monitoring changes in [Ca\textsuperscript{2+}]; in the rat epitrochlearis muscle by using the fluorescent indicator techniques, and, 2) using the new method, to assess the effect of several physiological (hypoxia) and pharmacological stimuli on [Ca\textsuperscript{2+}]; in rat skeletal muscle.

**MATERIALS AND METHODS**

**Materials.** Fura 2-AM was purchased from Molecular Probes (Eugene, OR). All other biochemicals were purchased from Sigma Chemical (St. Louis, MO).

**Animals and muscle preparation.** Male Sprague-Dawley rats (Kyudo, Kumamoto, Japan), each weighing 110–120 g, were used. All the animals were housed in rooms lighted from 7 AM to 7 PM and were maintained with ad libitum feeding on standard chow and water. The room temperature was maintained at 20–22°C. Food was restricted to 8 g on the evening of the last day before the experiment.

The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium, 5 mg/100 g body wt, and the epitrochlearis muscles were dissected out. The muscles weighed ~10–15 mg (wet weight) and averaged ~1.0 cm in length, 0.4 cm in width, and <0.2 mm in thickness (34). Approximately 80–85% of the epitrochlearis muscle fibers are composed of fast-twitch (type II) fibers (34). After dissection, the muscles were allowed to recover for >60 min in 25-ml stoppered Erlenmeyer flasks containing 3 ml oxygenated Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose and 32 mM mannitol. The gas phase in the flasks during the recovery period was 95% O\textsubscript{2}–5% CO\textsubscript{2} at 35°C.

**Fura 2 loading.** For fura 2 loading, the acetoxyethyl ester form of fura 2 (fura 2-AM) was used because it has the following properties: 1) Fura 2-AM is shown to be loaded into muscle cells easily in its cell-permeable AM form, which is deesterified by esterases in the cytoplasm. 2) Fura 2 alters its excitation spectrum on binding to Ca\textsuperscript{2+}. If we excite fura 2 by using two alternating wavelengths of light (typically 340 and 380 nm) while monitoring emissions near 500 nm, the ratio of fluorescence intensities at the two excitation wavelengths provides a measure of the [Ca\textsuperscript{2+}]; independently of the dye concentration (19).

First, fura 2-AM was dissolved in DMSO in a 2 mg/ml concentration, added to KHB to make a final concentration of 10 μM, and then mixed vigorously by applying ultrasonic waves. A noncytotoxic detergent, 0.02% Cremophor EL, was added to increase the solubility of the fura 2-AM (1).

The epitrochlearis muscles were incubated for 60–90 min at 35°C in 25-ml stoppered Erlenmeyer flasks containing 1.5 ml KHB with fura 2-AM. Because fura 2-AM is sensitive to light, the flasks were wrapped in aluminum foil. The gas phase in the flasks during the fura 2-AM loading period was 95% O\textsubscript{2}–5% CO\textsubscript{2}.

**Fluorescence measurement.** The fluorescence from the epitrochlearis muscle was measured with a fluorometer specially designed to measure the fluorescence of living tissues (CAF-110, Japan Spectroscopic, Tokyo, Japan) (Fig. 1) (37). Because previous studies showed that skeletal muscle tissue has a substantial amount of endogenous NADH, whose fluorescence has similar excitation and emission characteristics as that of fura 2-AM (1, 12, 13, 32, 37, 40), the fluorescence measured from fura 2-loaded muscle is thought to be a sum of fura 2-derived fluorescence and a non-fura 2 component of the pyridine nucleotides. Therefore, the fluorescence specific to fura 2 (F\textsubscript{fura,340} and F\textsubscript{fura,380}) was calculated as the difference between the fluorescence intensities from fura 2-loaded muscle (F\textsubscript{total,340} and F\textsubscript{total,380}) and those from fura 2-free muscle (F\textsubscript{fura-free,340} and F\textsubscript{fura-free,380}). To obtain F\textsubscript{total,340} and F\textsubscript{total,380}, one epitrochlearis muscle from a rat was used. The other epitrochlearis muscle from the same rat was used for the measurement of F\textsubscript{fura-free,340} and F\textsubscript{fura-free,380}. The difference in the two measurements was the fura 2-AM loading before the fluorescence measurement. To measure fluorescence, the muscle was held horizontally in a temperature-controlled organ bath filled with 5 ml oxygenated KHB. The proximal end of the muscle was connected to an isometric transducer (TB-651t, Nihon Kohden, Tokyo, Japan) to monitor muscle tension. The resting tension was adjusted to 5 × 10\textsuperscript{−2} N. The muscle was then incubated in the oxygenated KHB for 20 min at 35°C to remove unhydrolyzed fura 2-AM and to allow recovery from the stress incurred during the setting up. During the 20-min recovery period, 8 mM glucose were supplied with KHB to provide an energy supply for the muscles. The fura 2-free muscles were also incubated for 20 min by use of the same procedure as that for the fura 2-loaded muscles.

The light for excitation was emitted from a xenon high-pressure lamp (75 W) equipped with two grating monochromators (340 and 380 nm) by means of a chopping mirror. The intensities of the 340- and 380-nm light filter. The intensities of the two excitation wavelengths were measured near 500 nm, the ratio of the two excitation wavelengths provides a measure of the [Ca\textsuperscript{2+}]; independently of the dye concentration (19).

Experimental protocol. Experiment 1 was performed to confirm that fura 2-AM was successfully loaded into the rat epitrochlearis muscle. After fura 2-AM loading and 20 min of recovery in the organ bath, the muscles were incubated for 5
min with physiological saline containing 5 mM MnCl2 and 100 nM ionomycin. To avoid the precipitation of calcium in KHB by MnCl2, physiological saline was used for this specific experiment. The use of saline for incubating rat skeletal muscle with MnCl2 was previously validated by Tóth et al. (43). Ionomycin transports extracellular manganate ion (Mn2+) into cells (19). Mn2+ is used because the ion is known to bind to the fura 2 molecule and abolish the fluorescence emitted from fura 2 by transforming the fura 2 molecule (19). A stock solution of ionomycin was prepared in DMSO. MnCl2 and the stock solution of ionomycin were dissolved directly into physiological saline.

In experiment 2, the kinetics of $F_{\text{fura2,340}}$ and $F_{\text{fura2,380}}$ were evaluated. The ratio of $F_{\text{fura2,340}}$ to $F_{\text{fura2,380}}$ was calculated as $R$, and the change in the ratio from the baseline value ($\delta R$) was used as an index of the change in $[\text{Ca}^{2+}]$. Fluorescence was measured every 10 min during the 90-min incubation with oxygenated KHB containing 8 mM glucose and 32 mM mannitol without interventions, and $\delta R$ was calculated. At each time point, excitation was restricted to a short period (5 s) to avoid photobleaching of the fura 2-AM (6, 32) and to minimize the cytotoxic effects of the ultraviolet light (32).

In experiment 3, we observed the responses of $\delta R$ to pharmacological stimuli that are known to alter $[\text{Ca}^{2+}]$, in skeletal muscle. First, the muscles were incubated for 20 min with KHB containing 1, 3, 5, and 10 mM caffeine, which is known to release Ca2+ from the SR (36). Fluorescence was measured every 5 min, and the caffeine dose response and $\delta R$ were evaluated. Second, the effect on $\delta R$ of dantrolene, which is known to inhibit the release of Ca2+ from the SR in skeletal muscle (44), was observed. Muscles were incubated with 10 mM caffeine together with 25 mM dantrolene. Fluorescence was measured every 5 min for 20 min. Third, muscles were incubated for 20 min with 10 mM ionomycin, which transports extracellular Ca2+ into the cell (29). Fluorescence was measured every 5 min.

The purpose of experiment 4 was to establish the relationships between each of various stimuli that have been known to elevate glucose transport and $\delta R$. First, we observed the effect of hypoxia and subsequent reoxygenation on $\delta R$. The effect of the duration of hypoxia on $\delta R$ was evaluated by measuring the fluorescence every 10 min during a 60-min hypoxia, which was induced by incubating the epitrochlearis muscle in KHB gassed with 95% N2 and 5% CO2. To observe the effects of the different durations of reoxygenation on the $[\text{Ca}^{2+}]$, of the previously hypoxic muscle, $\delta R$ was calculated for the epitrochlearis muscles that were first exposed to hypoxic KHB for 10–30 min, followed by 10, 20, and 30 min incubation in the oxygenated KHB for reoxygenation of the muscle. Furthermore, we assessed the effect of dantrolene on $\delta R$ during hypoxia. Muscles were incubated with the hypoxic KHB containing 25 mM dantrolene for 20 min. Fluorescence was measured every 5 min. Second, the muscles were incubated for 20 min with KHB containing 50 mM W-7, which was first developed as a calmodulin antagonist. This concentration of W-7 is known to induce the release of Ca2+ from the isolated SR and to increase glucose transport activity in rat epitrochlearis muscle without decreases in high-energy phosphates and without visible tension development (46). The effect of 25 mM dantrolene on $\delta R$ during incubation with W-7 was also observed for 20 min. Third, we observed the effect of 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), an AMP-activated protein kinase (AMPK) activator. AICAR has been implicated in the stimulation of glucose transport through a mechanism similar to exercise/contraction (20). Muscles were incubated for 20 min with KHB containing 2 mM AICAR. Finally, muscles were incubated for 20 min with KHB containing 2 mM insulin and 0.1% radioimmunoassay-grade bovine serum albumin. This concentration of insulin is known to maximally stimulate glucose transport in rat skeletal muscle (11).

Data analyses. The R value was calculated as $F_{\text{fura2,340}}/F_{\text{fura2,380}}$. In addition, the change in $[\text{Ca}^{2+}]$, during each treatment was evaluated by observing the alteration of the $\delta R$ value, which was calculated as the difference between the pretreatment value of R and R at the specific time point of treatment. The pretreatment of R was defined as the value observed immediately before each treatment that started at least 20 min after the muscle was set in the organ bath circulated by the oxygenated KHB.

Statistics. All values were expressed as means ± SD. In experiment 1, statistical analysis was performed using a two-way ANOVA (Jandel Sigma Stat, Jandel, San Rafael, CA) to examine the effects of the fura-2 loading and MnCl2 + ionomycin incubation (fura 2 × MnCl2). Repeated measure-
ment of one-way ANOVA was used to evaluate the statistical significance in experiments 2 and 4. For analyzing the effects of caffeine concentration and incubation time (treatment × time), two-way ANOVA was performed in experiment 3. In all experiments, whenever the ANOVA indicated significant differences, the Tukey test was used for post hoc analysis. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Experiment 1. \( F_{\text{fura-free}}^{340} \) and \( F_{\text{fura-free}}^{380} \) were approximately one-half of \( F_{\text{total}}^{340} \) and \( F_{\text{total}}^{380} \), respectively (Table 1) \((P < 0.001)\). \( F_{\text{total}}^{340} \) and \( F_{\text{total}}^{380} \) after 5-min incubation with 5 mM MnCl\(_2\) and 100 nM ionomycin was reduced to a similar level that was not statistically different from \( F_{\text{fura-free}}^{340} \) and \( F_{\text{fura-free}}^{380} \), respectively, after the incubation with the same medium. Neither \( F_{\text{fura-free}}^{340} \) nor \( F_{\text{fura-free}}^{380} \) was affected by 5 min incubation with 5 mM MnCl\(_2\) and 100 nM ionomycin.

Experiment 2. Whereas both \( F_{\text{fura}}^{340} \) and \( F_{\text{fura}}^{380} \) decreased with time (Fig. 2A), \( \Delta R \) was stable for 60 min (Fig. 2B). However, after 70 min, \( \Delta R \) increased significantly \((P < 0.01)\).

Experiment 3. Incubation with 1 mM caffeine did not affect \( \Delta R \) (Table 2). Incubation with 3, 5, and 10 mM caffeine increased \( \Delta R \) significantly \((P < 0.05, 0.01, \text{and} \ 0.001, \text{respectively})\). The increment in \( \Delta R \) during the 20-min incubation with 10 mM caffeine was significantly greater than the increments observed during the 3 and 5 mM caffeine incubations \((P < 0.05)\), whereas no significant difference in \( \Delta R \) was observed between the 3 and 5 mM caffeine incubations.

As shown in Table 2, 25 \( \mu \)M dantrolene completely blocked the 10 mM caffeine-induced increment in \( \Delta R \).

Incubation for 20 min with 10 \( \mu \)M ionomycin elevated \( \Delta R \) (Table 2) \((P < 0.001)\). This increase in \( \Delta R \) was significantly greater than that observed in 10 mM caffeine-treated muscle \((P < 0.001)\).

Experiment 4. Hypoxia for 10 min in vitro resulted in a significant increase in \( \Delta R \) \((P < 0.05)\). Hypoxia for 60 min induced a further increase in \( \Delta R \) \((P < 0.01)\) (Fig. 3). The increased \( \Delta R \)s that were induced by 10 min and 20 min of hypoxia returned completely to the baseline value after 10 and 15 min of reoxygenation, respectively (Fig. 4). The 30 min hypoxia-induced increase in \( \Delta R \) also returned to the baseline value after 30 min of reoxygenation. However, it was still significantly higher after 20 min of reoxygenation \((P < 0.05)\). As shown in Fig. 5, 25 \( \mu \)M dantrolene completely blocked the hypoxia-induced increment in \( \Delta R \).

Incubation with 50 \( \mu \)M W-7 for 20 min significantly elevated \( \Delta R \) \((P < 0.05)\). The increase in \( \Delta R \) after W-7 incubation was completely blocked by incubation with 25 \( \mu \)M dantrolene (Fig. 6).

As shown in Table 3, no significant increase in \( \Delta R \) was observed during incubation for 20 min with 2 mM AICAR nor with 2 mU/ml insulin.

DISCUSSION

The present investigation demonstrated that, by using the fura 2-AM ratiometric fluorescence method, changes in \([\text{Ca}^{2+}]_i\) within the physiological range can be monitored in the rat epitrochlearis muscle. Furthermore, and to be more specific, the method revealed the

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**Table 1. Fluorescence intensities in rat epitrochlearis muscles before and after 5-min incubation with 5 mM MnCl\(_2\) together with 100 nM ionomycin**

<table>
<thead>
<tr>
<th></th>
<th>Fura 2-free Muscle</th>
<th>Fura 2-loaded Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F_{\text{fura-free}}^{340} )</td>
<td>( F_{\text{fura-free}}^{380} )</td>
</tr>
<tr>
<td>Before incubation</td>
<td>421 ± 102 (n=5)</td>
<td>399 ± 64 (n=5)</td>
</tr>
<tr>
<td>After incubation</td>
<td>414 ± 92 (n=5)</td>
<td>403 ± 65 (n=5)</td>
</tr>
</tbody>
</table>

Values are means ± SD, in arbitrary units; n, no. of muscles. \( F_{\text{fura-free}}^{340} \) and \( F_{\text{fura-free}}^{380} \), fluorescence intensities from fura 2-free muscles induced by 340- and 380-nm excitation lights, respectively; \( F_{\text{total}}^{340} \) and \( F_{\text{total}}^{380} \), fluorescence intensities of fura 2-loaded muscle. * Significant difference from the values obtained in fura 2-free muscles and those after incubation with MnCl\(_2\) and ionomycin at a level of \( P < 0.001 \).
respectively. f Significantly greater than baseline values of ratio of fura 2-specific fluorescence at 340-nm excitation to that at 380-nm excitation. a,b,c Significant increases after incubation at the condition described above at levels of P < 0.05, 0.01, and 0.001, respectively. * Significant differences from the values obtained in 10 mM caffeine-treated muscle at levels of P < 0.001.

Values are means ± SD; n, no. of muscles. ΔR, change from baseline values of ratio of fura 2-specific fluorescence at 340-nm excitation to that at 380-nm excitation. a,b,c Significant increases after incubation at the condition described above at levels of P < 0.05, 0.01, and 0.001, respectively. * Significant differences from the values obtained in 10 mM caffeine-treated muscle at levels of P < 0.001.

Fig. 3. Time course of the change in ΔR in response to in vitro hypoxia. Rat epitrochlearis muscles were incubated with Krebs-Henseleit buffer (KHB) gassed with 95% N2 and 5% CO2 for 60 min. Fluorescence intensity was measured every 10 min. Values are means ± SD for 6 muscles. Significant (+P < 0.05 and **+P < 0.01) differences from the values obtained before stimulation (0 min).

Fig. 4. Effects of reoxygenation on ΔR elevated by different durations of in vitro hypoxia. After 10–30 min of in vitro hypoxia, the hypoxic medium was replaced by oxygenated KHB to reoxygenate the hypoxic muscle. Values are means ± SD for 11–13 muscles. Significant (*P < 0.05 and **+P < 0.01) differences from the values obtained before in vitro hypoxia.

[Ca2+]i in the rat epitrochlearis muscle during hypoxia and subsequent reoxygenation. The result also demonstrated that insulin and AICAR have no effect on [Ca2+]i in rat skeletal muscle in vitro.

The calculated values of Ffura 340 and Ffura 2380 that represent the fluorescence specific to fura-2 were as great as Ffura-free 340 and Ffura-free 380 (Table 2). Furthermore, both Ftotal 340 and Ftotal 380 were reduced to the similar respective levels as were Ffura-free 340 and Ffura-free 380 (Table 1) by incubation with 5 mM MnCl2, which is known to abolish fluorescence from fura 2 (19). In other words, MnCl2 virtually quenched the fluorescence specific to fura 2. Therefore, these findings suggest that fura 2-AM was loaded into the rat epitrochlearis muscle and that Ffura 2340 and Ffura 2380 represented fluorescence specific to fura 2.

In agreement with the results of previous investigations (3, 40, 42), the pretreatment values of R observed in the present investigation varied substantially from preparation to preparation. Although the reason for this variation remains unknown, it seemed plausible that, in some muscles, a portion of fura 2-AM may enter SR and mitochondria, which contain large amounts of Ca2+ (27, 32). Therefore, in previous studies (28, 35, 40), not R but the ΔR value was used as an index of the change in [Ca2+]i during a treatment. In the present investigation, we also used ΔR as an index of the change in [Ca2+]i during each treatment in the rat epitrochlearis muscle.

ΔR was stable for 60 min during the resting condition, whereas a significant decrease was observed in both Ffura 340 and Ffura 2380. However, after 70 min, ΔR significantly increased (Fig. 2-B). This result suggests that fluorometric measurements of [Ca2+]i in rat epitrochlearis muscle in the present investigation is not reliable beyond 60 min. The possible reasons for the increase in ΔR after 70 min of incubation may be hydrolization, photobleaching, and decomposition of fura 2 during prolonged incubation (6, 38). Because Sato et al. (37) also concluded that fluorometric experiments on smooth muscle should be terminated within 60 min, incubation was also limited to 60 min in the present investigation.

In the present investigation, ΔR had the following characteristics (Table 2): 1) ΔR increased in response to caffeine (3–10 mM) in a concentration-dependent manner. 2) Dantrolene (25 μM) completely abolished the

Table 2. Effects of 20-min incubation with caffeine, dantrolene, and ionomycin on ΔR in rat epitrochlearis muscle

<table>
<thead>
<tr>
<th>Caffeine</th>
<th>1 mM</th>
<th>3 mM</th>
<th>5 mM</th>
<th>10 mM</th>
<th>10 mM + Dantrolene (25 μM)</th>
<th>10 μM Ionomycin</th>
</tr>
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<tbody>
<tr>
<td>(n = 6)</td>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>5 min</td>
<td>0.00 ± 0.05</td>
<td>0.10 ± 0.11*</td>
<td>0.13 ± 0.05b,d</td>
<td>0.35 ± 0.13c</td>
<td>−0.05 ± 0.08*</td>
<td>1.03 ± 0.46*</td>
</tr>
<tr>
<td>10 min</td>
<td>0.02 ± 0.06</td>
<td>0.11 ± 0.10a,d</td>
<td>0.15 ± 0.06b,d</td>
<td>0.34 ± 0.08c</td>
<td>−0.03 ± 0.12e</td>
<td>1.12 ± 0.56b</td>
</tr>
<tr>
<td>20 min</td>
<td>0.03 ± 0.09</td>
<td>0.13 ± 0.10a.d</td>
<td>0.18 ± 0.06b.d</td>
<td>0.30 ± 0.07c</td>
<td>−0.05 ± 0.12c</td>
<td>1.27 ± 0.73c</td>
</tr>
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</table>

* Significant (*P < 0.05, **P < 0.01, and ***P < 0.001) differences from the values obtained before in vitro hypoxia.
response of ΔR to incubation with 10 mM caffeine. 3) ΔR was elevated by Ca2+ ionophore (10 μM ionomycin) four times more than by 10 mM caffeine. These observations certify that, by using ΔR, the changes in [Ca2+]i induced by various pharmacological stimuli can be monitored in the rat epitrochlearis muscle.

Incubation of the epitrochlearis muscle with a hypoxic medium has been used to evaluate hypoxia-induced increases in glucose transport activity in skeletal muscle (7, 10, 46). Although previous investigations suggested that the elevation of [Ca2+]i might be involved in the mechanism related to in vitro hypoxia-stimulated glucose transport (7, 10, 46), until now no report has observed actual changes in [Ca2+]i in skeletal muscle during in vitro hypoxia. Using fluorometry of fura 2, the present investigation is the first to observe the effect of in vitro hypoxia on [Ca2+]i in skeletal muscle tissue.

In terms of glucose transport in skeletal muscle, because the maximal effects of in vitro hypoxia and muscle contraction were not additive (10), hypoxia was thought to stimulate glucose transport activity by the same pathway as muscle contraction. Although the mechanism by which hypoxia stimulates glucose transport is not known, the involvement of elevated [Ca2+]i after hypoxia has been suggested as a possible candidate (7, 10, 46). The present investigation showed that incubation for 10–20 min under a hypoxic condition resulted in significant increases in ΔR, a finding that suggests that [Ca2+]i does increase during in vitro hypoxia. Furthermore, a sustained elevation of ΔR (~0.20) was observed during 60 min of hypoxia.

When the glucose transport activity induced by submaximal (20 min) and maximal (60 min) durations of hypoxia was estimated, the activity had been actually measured after 15 min of reoxygenation, during which the ATP and creatine phosphate in the muscle were shown to recover (10, 41). The present investigation demonstrated that the elevated ΔR after 10- to 20-min hypoxia returned to the prehypoxia value after 15 min of reoxygenation. However, when the duration of hypoxia was prolonged to 30 min, ΔR did not recover to the pretreatment value after the same duration (15 min) of reoxygenation (Fig. 4). These results demonstrate that the longer the hypoxia, the more time it takes to restore [Ca2+]i afterward. Furthermore, the findings of the present investigation demonstrated that the glucose transport activity after 20-min hypoxia was measured without the elevation of [Ca2+]i. However, the results of this investigation also suggest that the glucose transport activity, which was measured by the normal procedure (15-min reoxygenation after 60-min hypoxia), and which is considered to represent the effects of 60-min hypoxia, might be measured in the condition with elevated [Ca2+]i (10). The involvement of [Ca2+]i in hypoxia-stimulated glucose transport in skeletal muscle has not yet been clarified. Furthermore, it is not known whether elevated glucose transport activity is a direct and short-lived effect of elevated [Ca2+]i, or an indirect and long-lived effect of a prior transient elevation of [Ca2+]i, for example, an aftereffect of calcium-dependent protein activation. If one attributes the hypoxia-stimulated glucose transport activity to a Ca2+-related mechanism, the results of the present investigation suggest that maximal hypoxia-stimulated glucose transport activity, normally measured after 15-min reoxygenation after 60-min hypoxia, might be regarded as a consequence of the

### Table 3. ΔR during 2.0 mM AICAR and 2.0 μU/ml insulin incubation of rat epitrochlearis muscle

<table>
<thead>
<tr>
<th></th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICAR (2.0 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 7)</td>
<td>-0.01 ± 0.03</td>
<td>-0.02 ± 0.05</td>
<td>0.01 ± 0.06</td>
</tr>
<tr>
<td>Insulin (2.0 μU/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 7)</td>
<td>-0.02 ± 0.02</td>
<td>-0.01 ± 0.02</td>
<td>0.01 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of muscles.
direct effects of the sustained elevation of \([Ca^{2+}]\) and the indirect biochemical effects related to the proceeding hypoxia-induced elevation of \([Ca^{2+}]\). However, as described above, only speculative discussion regarding hypoxia-stimulated glucose transport activity in rat skeletal muscle is available to date. We believe that, for the purpose of clarifying the relation between the hypoxia-induced increase in \([Ca^{2+}]\), and the hypoxia-stimulated glucose transport activity in rat skeletal muscle, information on \([Ca^{2+}]\), measured by using the method established in the present investigation, will be valuable.

\([Ca^{2+}]\), of epitrochlearis muscle did not change as a result of incubation with the maximal insulin dose (2 mU/ml) in terms of glucose transport activity. This result may suggest that insulin does not stimulate glucose transport through elevating \([Ca^{2+}]\). However, Bruton et al. (8) recently reported that, although insulin had no effect on the global myoplasmic \(Ca^{2+}\) concentration measured with a fluorescent indicator indo 1, the insulin-induced increase in \([Ca^{2+}]\) close to the membrane was observed by using the membrane-associating form of indo 1, FIP-18. Because a majority of fura 2 molecules in skeletal muscle fiber have been shown to be bound to relatively immobile myoplasmic constituents (25), fluorescence signals obtained from fura 2-loaded rat epitrochlearis muscle might not detect any change in membrane \([Ca^{2+}]\) but, instead, reflect the global free myoplasmic \(Ca^{2+}\) concentration, as with indo 1. Further studies are needed to examine the actual role and importance of insulin-mediated increases in membrane \([Ca^{2+}]\) in rat epitrochlearis muscle by using the membrane-permeable form of FIP-18.

The present investigation demonstrates that AICAR, which is known to activate AMPK, does not alter the \([Ca^{2+}]\). This finding suggests that changes in calcium concentration are not involved in the mechanism related to the enhancement of glucose transport activity by AMPK activation. AMPK activation and elevated \([Ca^{2+}]\), during muscle contraction have been postulated to mediate the contraction-induced increase in glucose transport activity (20, 45). Recently, Mu et al. (33) demonstrated that the stimulation of glucose transport was only partially blocked in electrically stimulated contracting muscle from mice expressing a kinase-inactive \(\alpha-2\) catalytic subunit of AMPK. The authors concluded that an AMPK-independent pathway must, in part, mediate the effect of contraction on glucose uptake in muscle. In this regard, the results of the present investigation might suggest that some portion of the enhanced glucose transport activity by contraction is induced by the calcium-independent pathway of AMPK activation. Furthermore, in the present investigation, a significant increase in \(\Delta R\) was observed during incubation with a low concentration of W-7 that was reported to stimulate glucose transport activity in rat epitrochlearis muscle without decreases in high-energy phosphates and visible tension development (45). These findings, including the results of the present investigation, may suggest that the contraction-induced increase in glucose transport activity is mediated separately by the activation of AMPK and the increase in \([Ca^{2+}]\). However, up to now, there has been no direct evidence that elevated \([Ca^{2+}]\) is involved in the contraction-stimulated glucose transport. Therefore, extensive studies are needed to further elucidate the complete mechanism underlying muscle contraction-stimulated glucose transport activity.

Monitoring \([Ca^{2+}]\) during skeletal muscle fiber contraction by using a fluorescence indicator has been shown to be sensitive to the changes in optical density induced by the tissue movement (9, 43). Also, in the present investigation, because of the movement of the epitrochlearis muscle during electrically stimulated muscle contraction, we could not measure, with certainty, the changes in \([Ca^{2+}]\), in response to the twitch and tetanic contraction of rat epitrochlearis muscle. Although Carroll et al. (9) suggested that stabilization of rat skeletal muscle fibers in agarose gel suspension is useful for excluding the movement of muscle during contraction, it is unknown whether the agarose gel suspension is applicable to rat epitrochlearis muscle. Future extensive studies are needed to establish a method for successfully monitoring \([Ca^{2+}]\) during muscle contraction in rat epitrochlearis muscle. Such a method would be a valuable tool for clarifying the mechanism of contraction-stimulated glucose transport activity in skeletal muscle.

In conclusion, the present investigation demonstrates that the physiological response of \([Ca^{2+}]\), in the rat epitrochlearis muscle to both pharmacological and physiological stimuli can be monitored by using the fluorometric method developed in the present investigation. Furthermore, the present investigation also suggests that the method will be valuable for studying the mechanism of the enhanced glucose transport activity of skeletal muscle in vitro.

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