Intracellular Calcium Signals Measured with Fura-2 and Aequorin in Frog Skeletal Muscle Fibers

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Abstract Intracellular Ca\(^{2+}\)-related optical signals during and after contraction (twitch and tetanus) were measured in single frog skeletal muscle fibers with fura-2 and aequorin. In twitch response, the peaks of [Ca\(^{2+}\)], estimated from the in vitro calibrations of fura-2 and aequorin were significantly different (0.5 \(\mu\)M for fura-2 and 5 \(\mu\)M for aequorin). Even 30s after twitch response, the fura-2 fluorescence ratio \(F_{340}/F_{380}\) signal did not recover to the resting level before stimulation. When the stimulation frequency was increased, an increase in the resting fura-2 ratio signal became obvious and after the cessation of stimulation this increase gradually recovered to the level before stimulation. After tetanus (50 Hz for 1 s), a higher fura-2 ratio signal than that before stimulation was sustained longer than 90 s. These results indicate that the dissociation of the released Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) binding sites probably takes longer time than that previously reported. The present results, therefore, demonstrate that fura-2 is advantageous for qualitative monitoring of a slight change in the resting level of [Ca\(^{2+}\)]. which is not easily detectable with aequorin. In addition, the problems encountered in quantitative estimation of [Ca\(^{2+}\)], with fura-2 are also discussed.

Key words: aequorin, fura-2, calcium transient, skeletal muscle.

In skeletal muscle fibers, the action potential triggers the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR), and the binding of Ca\(^{2+}\) to troponin elicits contraction. Parvalbumin, as well as troponin, binds to Ca\(^{2+}\) and acts as an intracellular Ca\(^{2+}\) buffer (Gosselin-Rey and Gerday, 1977; Gillis et al., 1982). After the increase in [Ca\(^{2+}\)], SR takes up Ca\(^{2+}\) again and Ca\(^{2+}\) is dissociated from the intracellular Ca\(^{2+}\) binding sites of troponin and parvalbumin. The time course of the return of Ca\(^{2+}\) to SR from parvalbumin is considered to be slower than that from troponin (Gillis et al., 1982; Cannel, 1986; Somlyo et al., 1985). The magnitude and the time course of contraction and Ca\(^{2+}\) transient might be altered.
when the contraction is elicited in the skeletal muscle fiber in which the released Ca$^{2+}$ does not completely dissociate from parvalbumin. For example, the twitch tension at several seconds after tetanic contraction is larger than that before stimulation (i.e. post-tetanic potentiation, see BLINKS et al., 1978). Caffeine at a low concentration, which is considered to elevate the resting [Ca$^{2+}$], also potentiates twitch tension (KONISHI and KURIHARA, 1987). Therefore, measurement of the resting level of [Ca$^{2+}$], is important to elucidate the movement of intracellular Ca$^{2+}$ after contraction and the effect of pre-saturation of Ca$^{2+}$ binding sites on tension development in skeletal muscle fiber.

The transient change in [Ca$^{2+}$], (Ca$^{2+}$ transients) during and after contraction has been measured using various intracellular Ca$^{2+}$ indicators (aequorin, metallochromic dyes, and purpurate dyes) (BLINKS et al., 1978; BAYLOR et al., 1982, 1985; KOVACS et al., 1983; MAYLIE et al., 1987; HIROTÄ et al., 1989). These indicators, however, are not sensitive enough to detect a slight change in the resting level of [Ca$^{2+}$]. Therefore, in the present study, the fluorescent Ca$^{2+}$ indicator dye fura-2, which has a high affinity for Ca$^{2+}$, was injected with aequorin into single skeletal muscle fibers, and the slight change in the resting level of [Ca$^{2+}$], during and after twitch and tetanic contraction was measured with the ratio of the fura-2 fluorescence intensities ($F_{340}/F_{380}$). Thus, a physiological significance of the change in the resting level of [Ca$^{2+}$], in skeletal muscle fiber will be discussed in relation to tension development.

The preliminary results of this study have been previously reported (KURIHARA et al., 1989; SUDA et al., 1989).

METHODS

_In vitro experiments._ In vitro experiments were conducted using glass capillary tubes. The internal diameter of the capillary tube used was 200 μm. Fura-2 fluorescence was measured with a fluorescence measuring system (CAM-200, JASCO, Japan) attached to an inverted microscope (TMD, Nikon, Japan). For the objective lens, a Fluor 20 (20 times magnification, N.A. = 0.75; Nikon, Japan) was used. The capillary was placed on the center of the objective lens. The incident light for excitation (340 or 380nm), emitted from a xenon lamp, was passed through a monochromator (10 nm bandwidth), and the light at both wavelengths was allowed to change at 1 kHz. The intensity of the excitation light was reduced using an N.D. filter (N.D. = 2) prior to entering the inverted microscope to minimize photobleaching of fura-2 (BECKER and FAY, 1987) and to avoid cell damage. The fluorescent light emitted from the excited dyes in the capillary tube was recorded through an interference filter (510 nm peak transmittance) which was placed between the collecting objective lens and the cathode of the photomultiplier. Fura-2 fluorescence was obtained by subtracting the dark current of the photomultiplier from the resultant fluorescence signals.

Solutions with various free-calcium ion concentrations ([Ca$^{2+}$]) for the in
vitro experiments were prepared at 20°C using Ca-EGTA [ethyleneglycol-bis (β-aminoethylyther)-N,N′-tetraacetic acid] solution (a slight modification of the method reported by HORIUTI, 1988). The composition of the Ca-EGTA solution was as follows (mM): CaCl₂ (BDH, U.K.), appropriate concentration at each pCa; KCl, 70; EGTA, 15; PIPES [piperazine-N,N′-bis (2-ethanesulfonic acid)], 6; fura-2, 0.05; and MgCl₂, 1-1.4. The pH was adjusted to 7.0 using KOH after the addition of fura-2, and the calculated ionic strength was 0.15 M. All of the inorganic salts used were reagent grade. [Ca²⁺] was calculated using a computer under the assumption that the apparent dissociation constant for the Ca-EGTA reaction at pH 7.0 was 393 nM (MARTELL and SMITH, 1974). The purity of EGTA (Dojin Chemicals Co. Ltd.), as confirmed by the pH metric method (MOISESCU and PUSCH, 1975), was 99%, and the concentration of nominally 1 mM fura-2 (Molecular Probes Inc., Eugene, OR, U.S.A.) was assumed to be 0.83 mM (BAYLOR and HOLLINGWORTH, 1988). For the pCa calculation, the amount of Ca²⁺ bound to fura-2 was taken into account under the assumption that the apparent dissociation constant of fura-2 for Ca²⁺ was 135 nM (GRYKIEWICZ et al., 1985). The data obtained from the solutions with a pCa value of less than 5.0 were unreliable and were not included in the calculation of the $K_{D,e}$ (the effective dissociation constant) by the least square method. A solution for saturating [Ca²⁺] (pCa 3.0) was prepared by simply adding CaCl₂ to the salt solution without EGTA (125 mM KCl, 10 mM PIPES, 1 mM MgCl₂, pH 7.0). The ratios of measured fluorescence intensity ($F_{340}/F_{380}$) in various pCa were fitted, using the least square method, to a simple 1:1 binding curve. The best-fit $K_{D,e}$ was calculated as follows:

$$K_{D,e} = \frac{[\text{Ca}^{2+}]}{(R_{\text{max}} - R/R - R_{\text{min}})} \left( \frac{S_2}{S_{f2}} \right),$$  

(1)

where $R_{\text{max}}, R_{\text{min}}$, and $R$ are the ratio values measured in solutions with saturated [Ca²⁺], no Ca²⁺ (EGTA solution, abbreviated here as 0 [Ca²⁺]), and in an intermediate [Ca²⁺], respectively. $S_2$ and $S_{f2}$ are proportionality coefficients which, when multiplied by the fura-2 concentration, yield the fluorescence levels at 380 nm excitation when all fura-2 is in the Ca²⁺-bound (S₂) and Ca²⁺-free (S₁) form (see GRYKIEWICZ et al., 1985). In practice, the value $S_2/S_{f2}$ was obtained using the value, $F(380, S)/F(380, 0); F(380, S)$ and $F(380, 0)$ represent fluorescence levels excited at 380 nm wavelength in the solutions containing saturated [Ca²⁺] and 0 [Ca²⁺], respectively. The relationship between pCa and the fura-2 ratio signal obtained from the in vitro measurement is shown in Fig. 1., where $R_{\text{max}} = 18.7$, $R_{\text{min}} = 0.507$, and $S_{f2}/S_2 = 13.9$ (fura-2 concentration, 50 μM). The best-fit $K_{D,e}$ of the theoretical curve in Fig. 1 was 162 nM. From the calculated $K_{D,e}$ (in the presence of Mg²⁺), $K_D$ (in the absence of Mg²⁺) could be calculated using the following equation (BAYLOR et al., 1982):

$$K_{D,e} = K_D (1 + [\text{Mg}^{2+}]/K_{D,Mg}).$$  

(2)

In the present experiments, [Mg²⁺] was 1 mM, and $K_{D,Mg}$ (apparent dissociation constant of fura-2 for Mg²⁺) was assumed to be 9.8 mM for the calculation.
Fig. 1. Relationship between pCa and fura-2 ratio signal of fluorescence ($F_{340}/F_{380}$) in a 200 $\mu$m diameter capillary tube. Since the fura-2 concentration was 50 $\mu$M, the inner filter effect was negligible. Open circles are experimental data and the data points were fitted using the least square method. $R_{max} = 18.7, R_{min} = 0.507$, and $S_{12}/S_{b2} = 13.9$. The best-fit $K_{D,e}$ was $162$ nM. For details, see METHODS section.

(Grynkiewicz et al., 1985). Thus, $K_D = 147$ nM was obtained. This $K_D$ value is in close agreement with the previously reported values (135 nM, Grynkiewicz et al., 1985; 161 nM, Kao and Tsien, 1988; 162 nM, Konishi et al., 1988).

In order to estimate the fura-2 concentration in fibers, calibration curves were made using capillary tubes by measuring the relationship between the fura-2 concentration and the fluorescent intensity measured at the isosbestic point (358 nm in our system).

Intact muscle fiber experiment. Single fast twitch muscle fibers were dissected from m. tibialis anterior of Rana temporaria which was kept at 4°C. One end of the preparation was attached to the hook and the other to the lever of a tension transducer with thin gold wires (diameter, 50 $\mu$m) to minimize movement during contraction which influences the fluorescence measurement. The transducer was composed of semiconductor elements (Kulite, New Jersey, U.S.A. or Akers, Horten, Norway). Preparations were horizontally mounted in a narrow channel (width, 3 mm) of a chamber with a pair of platinum black plates placed in parallel with the preparation for electrical stimulation. Sarcomere length of the fiber was adjusted to 2.4–2.5 $\mu$m by observing the laser (He-Ne laser, 1 mW) diffraction pattern. The length of the fiber was $6.5 \pm 0.7$ mm and the diameter was $145 \pm 59$ $\mu$m (mean $\pm$ standard error, S.E., $n = 18$). The cross-sectional area was calculated assuming an ellipsoidal shape, and the developed tension was normalized to the cross-sectional area. Fiber condition was checked before and after injection of $\text{Ca}^{2+}$ indicators (aequorin and/or fura-2) by observing tetanic contraction elicited by 20 square pulses at 50 Hz. Fibers that showed complete tetanus with a sustained
plateau were used for experiments. The temperature of the solution was continuously monitored with a thermocouple with a 0.1 s time constant and maintained at 18°C. The preparation was stimulated with 500 μs square pulses at a 1.5-fold threshold.

Normal Ringer solution was composed of the following (mM): NaCl, 115; KCl, 2.5; CaCl₂, 1.8; 3-(N-morpholino) propanesulfonic acid (MOPS), 5.0; the pH was adjusted to 7.0 with NaOH.

Aequorin (purchased from Dr. J. R. Blinks, Mayo Foundation) was dissolved in the 150 mM KCl and 5 mM HEPES (N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid) solution, pH 7.0, to a final concentration of 50–100 μM (1–2 mg/ml). For injection of aequorin, the tip of a glass micropipette (electrical resistance, 20–30 MΩ) which was fixed in an injection device was filled with a small amount of the solution. After confirming penetration of the micropipette into the fiber, aequorin was injected by carefully applying pressure (5–7 kg/cm²) while monitoring the membrane potential to avoid fiber damage. After completion of injection, the light guide (1 cm in diameter), which was connected to a photomultiplier (EMI 9789 A, Ruislip, U.K.) within a housing, was placed just above the fiber for the detection of the resultant light. For conversion of the aequorin light signal to [Ca²⁺], the light signal was expressed as fractional luminescence (L₀, logarithm of a light signal normalized to maximal light, Allen et al., 1977). For details of the procedure, see Allen and Kurihara (1982). The in vitro calibration curve was determined at 18°C and the data were fitted using the least square method using the following equation:

\[
\frac{L}{L_{\text{max}}} = \left( \frac{1 + K_R[Ca^{2+}]}{1 + K_{TR} + K_R[Ca^{2+}]} \right)^n,
\]

where \(L/L_{\text{max}}\) is the normalized aequorin light signal divided by the maximum light emission of injected aequorin (obtained at the end of the experiment), and \(n, K_R, K_{TR}\) are constants in the 2 state model (see Allen et al., 1977). The following constants were used: \(n = 3.22, K_R = 4,776,000, K_{TR} = 116.3\) [Mg²⁺], was assumed to be 1 mM.

The following solution was used for fura-2 injection (mM): KCl, 100; MOPS, 5; fura-2 penta-potassium salt, 2.5–10, pH 7.0. The method of fura-2 injection was the same as that for aequorin and the detection of fluorescent light was also the same as described in the in vitro experiment with fura-2, although the artifactual fluorescent signal from the non-fura-2 component (background fluorescence primarily from chamber) could not be completely eliminated. When the fluorescence of fura-2 was measured from single muscle fibers, fura-2 was injected into several points near the tendon where movement during contraction was minimal. The calibration bars in the figures of the present in vivo experiment are not corrected for the non-fura-2 component. (Fluorescence from the chamber and cell derivative auto-fluorescence. This component was less than 8% of the resting fluorescence intensity at 340 and 380 nm excitation. However, during tetanus, it was less than 22% of the fluorescence intensity at 380 nm excitation.)
calculation of $[\text{Ca}^{2+}]$, from the fura-2 ratio signal, however, the non-fura-2 component was subtracted from the recorded fluorescence.

All data were stored on a tape recorder (NFR-3515W, Sony Magnescale Inc., Tokyo, Japan) and replayed later for analysis.

Data were expressed as mean ± standard error (S.E.).

RESULTS

Fura-2 signal during rest and twitch responses

Fura-2 ratio signal in frog skeletal muscle fibers at the resting state was between 0.37 and 0.5. When the ratio value was simply converted to $[\text{Ca}^{2+}]$, using the pCa-ratio curve obtained from the in vitro measurement (Fig. 1), the corresponding $[\text{Ca}^{2+}]$, had a negative value which was consistent with the results of a cut fiber experiment reported by KLEIN et al. (1988). This discrepancy indicates that $R_{\text{min}}$ is suppressed in the myoplasm and supports an earlier report (KONISHI et al., 1988) that the absorbance spectrum of the $\text{Ca}^{2+}$-free form of fura-2 shows a red shift when fura-2 binds to myoplasmic soluble proteins.

The ratio signal in response to a single action potential is shown in Fig. 2 with the corresponding fluorescence changes at 340 and 380 nm excitation and tension.

![Fig. 2. Simultaneous records of fura-2 signals and tension in twitch response. In A, the fluorescent signal at excitation wavelengths of 340 and 380 nm (upper two records), and the ratio signal (middle trace). The bottom trace is tension. In B, movement artifacts were checked by measuring the fluorescence intensity at 358 nm excitation. In each record, two signals were averaged. Sarcomere length was 2.4 μm. The stimulus is indicated as small upward deflections on the lower trace. Notice that the scale of the fluorescence trace of each is arbitrary (output voltage of PMT amplifier) in this figure and others.](image)
The amount of fura-2 injected was estimated to be 80 \( \mu \text{M} \). When the fiber was stimulated, the fluorescence intensity at 340 nm excitation was increased, and that at 380 nm was decreased with a similar time course. The ratio signal increased and reached the peak 22 ms after stimulation, and then decayed exponentially (half decay time, 27 ms). The ratio signal at 200 ms after stimulation was higher than that before stimulation and this increase was detectable even 30 s after stimulation (see Fig. 5A). The fluorescence intensity at 358 nm excitation did not change during contraction, indicating that there was no involvement of movement artifacts in the fluorescence measurement. \([\text{Ca}^{2+}]\), at the peak of the ratio signal in twitch response from several fibers was 545 \( \pm \) 153 nM (80 \( \mu \text{M} \) fura-2 was injected into these fibers to avoid the inner filter effect and \( \text{Ca}^{2+} \) buffering effect of fura-2) \((n = 6)\), when the ratio values were directly converted to \([\text{Ca}^{2+}]\), using the \textit{in vitro} calibration and assuming instantaneous equilibration of fura-2 with \( \text{Ca}^{2+} \). This value was smaller than that estimated with aequorin (5 \( \mu \text{M} \)). Time to peak ratio signal was 18.0 \( \pm \) 1.2 ms \((n = 10)\). The decay of the ratio signal from the peak showed two phases (Figs. 2 and 5A). The early phase (the first 500 ms after the cessation of stimulation) decayed exponentially with a half decay time of 23.9 \( \pm \) 2.6 ms \( (n = 11) \). The later phase decayed rather linearly with a half decay time of 51.4 \( \pm \) 6.9 s \((n = 11)\) (see Fig. 5).

Changes in the resting fura-2 signals after stimulation at different frequencies

Figure 3 shows the effects of stimulation frequency on the ratio signal of fura-2 and tension in twitch response. At 1/1 s, the peak of twitch tension initially diminished and then gradually increased. This was obvious at higher frequencies (1/0.5 and 1/0.3 s; Fig. 3C and E). An obvious change in the fura-2 ratio signal was the increase in the basal level (the resting ratio signal). The increase in the resting ratio signal was larger when the stimulation frequency was higher. The involvement of movement artifacts in the change of the fura-2 ratio signal was checked by measuring the fluorescence intensity at 358 nm excitation (Fig. 3B, D, F). No remarkable change in the resting fluorescence intensity at 358 nm excitation was observed at any of the stimulation frequencies used, although slight negative deflections corresponding to each stimulation were observed. Furthermore, a similar increase in the resting ratio signal was also observed when the fiber was stretched to a sarcomere length of 3.6 \( \mu \text{m} \) (data not shown). Therefore, the increase in the resting ratio signal was not due to movement of the fiber during contraction and reflects a change in resting \([\text{Ca}^{2+}]\).

Fura-2 signals in tetanus

Fura-2 ratio signal and fluorescence intensities at 358, 340, and 380 nm excitation in tetanic stimulation were recorded (50 Hz for 1 s) (Fig. 4). In Fig. 4A the sarcomere length of the fiber was set at 2.6 \( \mu \text{m} \). The ratio signal increased during stimulation and finally reached a steady level. As in the case of twitch response, fluorescence intensities at 340 and 380 nm excitation changed in the
Fig. 3. Fura-2 ratio signals in twitch responses at different stimulation frequencies. In A, C, and E, the upper trace is the ratio signal of fura-2 and the middle trace is tension. The bottom trace in each record is stimulus. The stimulation frequency was changed as follows: A, 1/1 s; C, 1/0.5 s; E, 1/0.3 s. The number of stimulations was 30 pulses in each case. In B, D, and F, movement artifacts were checked at each stimulation frequency by measuring the fluorescence at 358 nm excitation wavelength. B, 1/1 s; D, 1/0.5 s; F, 1/0.3 s.

opposite direction. The slight change in the fluorescence intensity at 358 nm excitation indicated that there were slight movement artifacts involved in the fluorescence intensities at 340 and 380 nm excitation and consequently in the ratio signal. The change in the fluorescence intensity at 358 nm excitation was completely eliminated by stretching the fiber to a sarcomere length of 3.6 μm (Fig. 4B). At a sarcomere length of 3.6 μm, tetanic tension was less than that at 2.6 μm and the changes in the fluorescence intensity and the resultant ratio signals were diminished. [Ca^{2+}]i at 1 s after starting tetanic stimulation, as estimated from the
Fig. 4. Fura-2 signals in tetanic contraction at different sarcomere lengths. In A, the sarcomere length was 2.6 \( \mu \text{m} \) and in B, the preparation was stretched to 3.6 \( \mu \text{m} \). In each record, the fluorescence at each excitation wavelength (358, 340, 380 nm) and ratio (\( F_{340}/F_{380} \)) were measured with tension. The bottom trace is stimulus (50 Hz for 1s). A slight fluorescence change at 358 nm excitation was observed at sarcomere length 2.6 \( \mu \text{m} \) and this disappeared when the fiber was stretched to 3.6 \( \mu \text{m} \). At 3.6 \( \mu \text{m} \), tension increased at a slower rate than that at 2.6 \( \mu \text{m} \). Changes in fluorescent signal and ratio were reduced at 3.6 \( \mu \text{m} \).

peak of the ratio signals using the \textit{in vitro} calibration, was 1,120±320 nM \( (n=20) \) at 2.4 \( \mu \text{m} \), and 851±19.6 nM \( (n=3) \) at 3.6 \( \mu \text{m} \). On the other hand, that measured with aequorin was 6.02±1.3 \( \mu \text{M} \) at a sarcomere length of 2.4 \( \mu \text{m} \) \( (n=6) \). The ratio signal following the tetanus showed two phases of decay. The half decay time of the ratio signal during the first phase (the first 1 s after the cessation of stimulation) was 66.8±9.1 ms \( (n=13) \). During the second phase, the ratio value was much larger than that observed in twitch and was appreciably larger than the resting level even 90 s after the cessation of stimulation (Fig. 5). The slow return of the fura-2 ratio signal was also observed at a sarcomere length of 3.6 \( \mu \text{m} \), where movement artifacts could completely be neglected. Therefore, the slow return of the fura-2 ratio signal at sarcomere length of 2.4 \( \mu \text{m} \) is not due to the movement of the fiber and might reflect the slow decay of \( \text{Ca}^{2+} \).

\textit{Aequorin light signals in twitch response before and after injection of fura-2}

Since fura-2 has a high affinity for \( \text{Ca}^{2+} \), there is a possibility that a large dose of fura-2 in the myoplasm acts as an intracellular \( \text{Ca}^{2+} \) buffer. In order to confirm
Fig. 5. The resting level of the fura-2 ratio signals after twitch (closed circles) and tetanus (open circles). In twitch, each circle represents the average value of 10 records from two fibers and in tetanus, 7 signals from the same fiber. Standard error is enclosed within each circle. Note that the ratio signal at 30 s after the twitch response or 90 s after the tetanus was still higher than that before stimulation. Inset shows typical records of the fura-2 ratio signal after twitch (A) and tetanus (B). Both traces were recorded at high gain through a 1 Hz low-pass filter, and the top of both records was overscaled. Dotted line represents the resting level of the ratio signal before stimulation. Notice the different scales of ΔRatio in twitch and tetanus.

The Ca²⁺ buffering action of fura-2, the aequorin signals before and after fura-2 injection were compared (5 experiments). The concentration of fura-2 injected was estimated from the fluorescence intensity at 358 nm excitation (see METHODS). Figure 6 shows the effects of 80 μM fura-2 injection on aequorin light transients and tension. It is apparent that the peak amplitude of the aequorin light signal was slightly diminished after injection of fura-2. The same results were obtained in 5 fibers into which fura-2 at a concentration of greater than 80 μM was injected. The peak amplitude of aequorin light transients and tension was reduced dose-dependently. They were more reduced at 720 μM than at 80 μM and were not significantly reduced at 10 μM. Injection of solution with 150 mM KCl (pH 7.0 buffered with HEPES) did not alter the aequorin light signal and tension. The results suggest that injection of fura-2 at a concentration of greater than 80 μM increases the intracellular Ca²⁺ buffer and reduces the amplitude of Ca²⁺ transients and

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**DISCUSSION**

1. **Problems of quantitative estimation of $[\text{Ca}^{2+}]$ with fura-2.** Fura-2 has been widely used in various types of cells to estimate $[\text{Ca}^{2+}]$, change. However, the fluorescence measurement method has the following difficulties compared to the aequorin method: 1) excitation by ultraviolet light damages cells; 2) photobleach-
ing of fluorescent dyes; 3) movement of the preparation influences fluorescence (movement artifact); 4) binding of dyes to the cellular component changes the properties of the dyes; 5) high optical density influences fluorescence intensity (the inner filter effect). For the most precise measurement possible, it is necessary to consider these problems in the measurement of the fura-2 ratio signal in single skeletal muscle fibers.

The damage to the fiber can be minimized by reducing the intensity of the excitation light using an N.D. filter, thus in the present experiment no obvious damage to the fiber was observed. Photobleaching was also eliminated by exposing the fibers to excitation light for a short time on recording. Since, in the present study, the both ends of the preparation were tightly fixed with thin gold wires, the fura-2 ratio signal was not significantly influenced by muscle contraction (Figs. 2–4), which was confirmed by the measurement of the fluorescence intensity at 358 nm excitation (isosbestic point).

The underestimation of \([Ca^{2+}]_i\) with fura-2 ratio signal using the \textit{in vitro} calibration, slower time course of fura-2 \(Ca^{2+}\) transient than that expected from the \textit{in vitro} data, and the evidence that the resting \([Ca^{2+}]_o\) had a negative value if the \textit{in vitro} calibration was directly applied to estimate \([Ca^{2+}]_i\), all suggest that the properties of fura-2 in myoplasm differ from those in a cuvette.

There are two possible explanations for underestimation of \([Ca^{2+}]_i\) in muscle fiber estimated from the fura-2 ratio signal using the \textit{in vitro} calibration: 1) increased myoplasmic viscosity, 2) binding of fura-2 to myoplasmic soluble proteins. POENIE \textit{et al.} (1986) suggested that an increased myoplasmic viscosity could explain the reduction in the ratio value compared to that measured in a salt solution. Furthermore, KONISHI \textit{et al.} (1988) by steady state anisotropy measurement, indicated that as much as 80% of fura-2 molecules in myoplasm are in protein-bound form, and that binding alters many properties of the dye; there is an increase in \(K_D\) of fura-2 to \(Ca^{2+}\) as well as the red shift in the absorbance spectrum of the \(Ca^{2+}\)-free form of fura-2. Therefore, these two factors are probably related to the negative value of the resting \([Ca^{2+}]_i\) and the underestimation of peak \([Ca^{2+}]_i\) in twitch or tetanus. However, there is another possibility that \(R_{max}\) is reduced in myoplasm. The considerably small \(R\) in high \(K^+ (83 \text{ mM})\) contracture (6.69 ± 0.8) \((n = 3)\), where developed tension is nearly maximum, favors this hypothesis. The result that an increase in fura-2 concentration reduced the ratio signal at high \([Ca^{2+}]_i\) measured in a capillary tube (Fig. 7 in APPENDIX) suggests that the same reduction in the ratio signal at higher \([Ca^{2+}]_i\), occurred in muscle fibers. For example, if the average path length of the excitation light is 157 \(\mu m\) and the injected fura-2 concentration is 400 \(\mu M\), \(R_{max}\) is reduced by 17.6% estimated from Eq. (5) in the APPENDIX (in this calculation we assumed that the molar extinction coefficients of the \(Ca^{2+}\)-bound form of fura-2 in the myoplasm are the same as those in a salt solution). Furthermore, there exists a large intrinsic absorbance of the excitation light by muscle fibers. This factor further reduces \(R_{max}\) by 3.2% if the average path length of the muscle fiber is 157 \(\mu m\) and injected fura-2 concentration.

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is 400 nM (Eq. (12) in APPENDIX). Therefore, as a total, 20.2% (17.6 + 3.2 × 0.82) is reduced by these two factors in this case. In addition, the possibility that a relative reduction in the quantum yield of the Ca^{2+}-bound form of fura-2 occurred in myoplasm at 340 nm relative to that at 380 nm cannot be excluded. However, in the present experiment, this hypothesis was not further investigated.

2. Physiological significance of an increase in [Ca^{2+}], after contraction. When the action potential triggers the release of Ca^{2+} from SR, Ca^{2+} binds troponin and parvalbumin. The released Ca^{2+} is removed by the active pump of SR, and Ca^{2+} bound to troponin and parvalbumin dissociates from its binding sites. Although the rate of dissociation of Ca^{2+} from troponin is fast, that from parvalbumin is rather slow (GILLIS et al., 1982). Since the concentration of parvalbumin is about 0.4 mM (GOSSelin-ReY and GERDAY, 1977), a change in the Ca^{2+} binding capacity of parvalbumin significantly influences the time course of the falling phase of the Ca^{2+} transients. Thus, pre-saturation of the Ca^{2+} binding sites of parvalbumin is considered to influence Ca^{2+} transients and developed tension.

When the stimulation frequency was increased, the resting level of the fura-2 ratio signal increased during stimulation (Fig. 3). Detection of a similar elevation of the resting light signal of aequorin is difficult due to low sensitivity of aequorin to Ca^{2+}. In addition, the peak of the fura-2 ratio signal was increased during repetitive stimulation. This contradicts the results observed using other indicators (BLINKS et al., 1978; KONISHI and KUHIRA 1987; BAYLOR and HOLLINGWORTH, 1988; HIROTA et al., 1989). An increase in the peak of the fura-2 ratio signal during repetitive stimulation might be caused by the slower decay of the fura-2 ratio signal (Figs. 2, 5). If the fura-2 ratio signal, triggered by the action potential, is superimposed on the elevated falling phase of the preceding signal, the peak of the fura-2 ratio signal is expected to be increased. This speculation is confirmed by the fact that during the first several stimulations the peak of the fura-2 ratio signal did not change, rather it increased, although tension gradually decreased (Fig. 3C, E).

Following tetanic contraction, the muscle relaxed in two phases (shoulder and exponential relaxation) and the corresponding [Ca^{2+}], change, measured with aequorin, is divided into 3 periods (CANNELL, 1986). In period 3 when the muscle relaxes, [Ca^{2+}], does not completely recover to the resting level before stimulation. However, the low [Ca^{2+}], at the later stage of period 3 could not accurately be detected with aequorin as [Ca^{2+}], is very close to the detection limit of aequorin. Therefore, injection of a large amount of aequorin and signal averaging are necessary to measure the low [Ca^{2+}], with aequorin. Moreover, aequorin light signal after tetanic contraction might be influenced by an increase in [Mg^{2+}], because Mg^{2+} dissociates from its binding site of parvalbumin in exchange for Ca^{2+} binding. Therefore, the quantitative estimation of [Ca^{2+}], with aequorin after tetanus also difficult in this respect. However, in the present experiment, the fura-2 ratio signal, which is influenced to a lesser extent by [Mg^{2+}], showed an obvious increase in the resting [Ca^{2+}], even 90 s after tetanus. Similarly, the resting [Ca^{2+}],
was higher than that before stimulation even 30 s after twitch response (Fig. 5). These results indicate that the dissociation of Ca\(^{2+}\) from parvalbumin after tetanus is considerably slow and takes more than 1 min, although the recovery of Ca\(^{2+}\) content in SR has been reported to be completed within a few seconds (SOMLYO et al., 1985).

The sustained elevation of [Ca\(^{2+}\)], after tetanic contraction probably related to post-tetanic potentiation. If Ca\(^{2+}\) does not completely dissociate from the Ca\(^{2+}\) binding sites of parvalbumin, the capacity of Ca\(^{2+}\) buffer is reduced. Then, the reduced Ca\(^{2+}\) buffering capacity prolongs the duration of Ca\(^{2+}\) transients and thus potentiates twitch tension even if the amount of the released Ca\(^{2+}\) is decreased (BLINKS et al., 1978). On the other hand, the result that the injection of a high dose of fura-2, which is considered to increase the intracellular Ca\(^{2+}\) buffering capacity, reduced the peak and duration of Ca\(^{2+}\) transient (Fig. 6) confirms the report by BAYLOR and HOLLINGWORTH (1988), which showed a change in Ca\(^{2+}\)-related antipyrlylazo III signals by fura-2 injection.

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REFERENCES


APPENDIX

The purpose of appendix section 1 is to show that $R_{\text{max}}$ is reduced by the inner filter effect (CANTOR and SCHIMMEL, 1980) and to estimate the degree of reduction in $R_{\text{max}}$ in muscle fibers when the fiber is injected with a large dose of fura-2. Appendix section 2 gives rough estimate of the influence of fiber intrinsic absorbance on $R_{\text{max}}$.

1. Evaluation of the influence of the inner filter effect on the ratio signal of fura-2

The influence of the fura-2 concentration on the ratio value of fluorescence was examined in a glass capillary tube using the same Ca$^{2+}$ concentration and different

![Fig. 7. Effects of fura-2 concentration on the ratio signal of solutions at pCa 3.0 (open circles), pCa 6.0 (squares), and pCa 7.7 (triangles). All measurements were made in a capillary (internal diameter: 200 μm). Fura-2 concentration was increased from 50 to 300 μM. At higher fura-2 concentrations, the ratio at pCa 3.0 and pCa 6.0 decreased but that at pCa 7.7 did not change. The theoretical values (closed circles) are based on Eq. (7). Since the theoretical absolute values are influenced by $q$ (the ratio of the quantum yield of Ca$^{2+}$-bound form of fura-2 to that of Ca$^{2+}$-free form), the theoretical values at pCa = 6.0 become more close to the measured values if we use 1.8 instead of 2.0 as the value of $q$. For details of composition of the solutions used, see METHODS section.](image-url)
fura-2 concentrations (50–300 μM). Figure 7 shows the results obtained using the solutions with a pCa value of 7.7 (triangles), 6.0 (squares), and 3.0 (circles). It is obvious that the ratio values at pCa 3.0 and 6.0 decreased as the fura-2 concentration was increased. On the other hand, the ratio values at pCa 7.7 were not altered.

Prior to the assessment of the inner filter effect, the effective focal depth for the fluorescence was estimated, using capillary tubes with different internal diameters (d = 25, 30, 50, 75, 100, 125, 150, 200, 300 μm), each containing either 2,000 or 100 μM fura-2 and the fluorescence intensities (F) at 340 and 380 nm excitation were measured. The effective focal depth was estimated to be approximately 80 μm judging from the relation between the fluorescence intensity and the internal diameter of the capillary (KLEIN et al., 1988).

From Beer-Lambert's law, the intensity of the incident light of excitation wavelength at distance X from the internal surface of the capillary is given by

\[ I_{\text{in}}(X) = I_o 10^{-EXX} \]

where \( I_o \) is the intensity of the incident light entering the internal surface of the capillary, \( E \) is the molar extinction coefficient of fura-2 at the excitation wavelength, and \( C \) is the concentration of fura-2. Under the assumption that the steady state fluorescence intensity is proportional to the incident light absorbed by fura-2 within the effective focal depth (\( D \)) for the fluorescence,

\[ F = \phi I_o [10^{-E(p-D)2} - 10^{-E(p+D)2}] \]

\[ = \phi I_o (1 - 10^{-EC(p-D)2}) 10^{-E(p-D)2} \]

where \( \phi \) is the proportionality constant that is dependent on the quantum yield of fura-2 at the excitation wavelength and on geometrical factors, and \( p \) is the average path length of the incident light (\( p = \pi d/4 \)). Note that Eq. (5) is available when \( p \) is larger than \( D \). If \( p \) is smaller than \( D \), the fluorescence intensity is given by

\[ F = \phi I_o (1 - 10^{-ECp}) \]

If we assume that quantum yield of Ca\(^{2+}\)-bound form of fura-2 is \( q \) times larger than that of Ca\(^{2+}\)-free form at all wavelengths, \( R \) at a given [Ca\(^{2+}\)] can be calculated by using the following equation:

\[ R = \left(I_{o,340}/I_{o,380}\right) \left[I_{o,380}/I_{o,380}\right] \left[q(1 - 10^{-E(380,S)/CD}) 10^{-E(340,S)/C(p-D)2} \right. \]

\[ + (1 - 10^{-E(340,0)(1-f)/CD}) 10^{-E(340,0)(1-f)/C(p-D)2}) \right. \]

\[ \left. - 10^{-E(380,0)/CD} 10^{-E(380,0)/C(p-D)2} + (1 \right. \]

\[ \left. - 10^{-E(380,0)(1-f)/CD}) 10^{-E(380,0)(1-f)/C(p-D)2}) \right]. \]

The notations in Eq. (7) are as follows: \( I_{o,340}, I_o \) of 340 nm wavelength; \( I_{o,380}, I_o \) of 380 nm wavelength; \( \phi_{340,0}, \phi \) of Ca\(^{2+}\)-free form of fura-2 at 340 nm wavelength; \( \phi_{380,0}, \phi \) of Ca\(^{2+}\)-free form of fura-2 at 380 nm wavelength; \( f \), fraction of Ca\(^{2+}\)-bound form of fura-2 \([f = 1/(1 + K_{0.2}/[Ca^{2+}]])\]; \( E(340, S) \), \( E \) of Ca\(^{2+}\)-bound form of fura-2 at 340 nm wavelength; \( E(340, 0) \), \( E \) of Ca\(^{2+}\)-free form of fura-2 at 340 nm wavelength.
wavelength; \( E(380, S) \), \( E \) of Ca\(^{2+}\)-bound form of fura-2 at 380 nm wavelength; \( E(380, 0) \), \( E \) of Ca\(^{2+}\)-free form of fura-2 at 380 nm wavelength.

Following to Eq. (7), \( R(p\text{Ca}=3.0) \) is reduced by 10\% and \( R(p\text{Ca}=6.0) \) is reduced by 8\% when the fura-2 concentration is increased from 100 to 300 \( \mu \text{M} \) in a 200 \( \mu \text{m} \) capillary tube. On the other hand, \( R(p\text{Ca}=7.7) \) is increased only by 3\%. These values are in good agreement with the measured values and are shown with closed circles in Fig. 7. To give the absolute values, apparatus-dependent value in Eq. (7) was estimated from the measured value of \( R(p\text{Ca}=3) \) at 50 \( \mu \text{M} \) fura-2. In these calculations, we assumed that the quantum yield of Ca\(^{2+}\)-bound form of fura-2 is 2 times larger than that of Ca\(^{2+}\)-free form (Grynkievicz et al., 1985). The following values were used in calculations: \( p=157 \mu \text{m} \), \( D=80 \mu \text{m} \), \( E(340, 0) = 1.9 \times 10^{5} \text{ M}^{-1} \cdot \text{cm}^{-1} \), \( E(340, S) = 3.2 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \), \( E(380, 0) = 2.4 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \), \( E(380, S) = 0.1 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \). Since the absolute value of \( E(362, 0) = 2.8 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \) (Grynkievicz et al., 1985; Baylor and Hollingworth, 1988), the absolute value of \( E(\lambda) \) could be calculated by our measured absorbance data \([A(\lambda)]\) using the following equation:

\[
E(\lambda) = E(362, 0) \times A(\lambda)/A(362, 0).
\] (8)

2. Evaluation of the influence of the intrinsic absorbance (and the inner filter effect) on the fura-2 ratio signal

Denote the intensity of the excitation incident light entering the surface of the fiber by \( I_{e} \). From Beer-Lambert's law, the intensity of light at distance \( X \) from the surface of the fiber \([I_{e}(X)]\) is given by

\[
I_{e}(X) = I_{e} 10^{-(t+EC)X},
\] (9)

where \( t \) is the fiber intrinsic absorbance per unit length, \( E \) is the molar extinction coefficient of the dye, and \( C \) is dye concentration in the fiber. The absorbed light by the dye within the slab at \( X \) \([\Delta I_{d}(X)]\) is given by differential form of Eq. (9) when multiplied by a fraction of dye absorbance to total absorbance \([EC/(t+EC)]\).

\[
\Delta I_{d}(X) = EC(\ln 10)I_{e}10^{-(t+EC)X}DX.
\] (10)

Total absorbed light by the dye \((I_{d})\) within the effective focal depth \((D)\) could be obtained by integrating \(\Delta I_{d}(X)\) over the effective focal depth along the optical axis.

\[
I_{d} = EC(\ln 10)I_{e}\int_{0}^{D}10^{-(t+EC)X}dX
= [EC/(t+EC)]I_{e}[10^{-((t+EC)(p-D)/2)} - 10^{-(t+EC)(p+D)/2}]
\] (11)

where \( p \) is the average path length. Therefore, if we assume that the measured steady state fluorescence intensity \((F)\) is proportional to \( I_{d} \), \( F \) is given by

\[
F = \phi[EC/(t+EC)]I_{e}[1 - 10^{-(t+EC)p}]10^{-(t+EC)(p-D)/2}.
\] (12)

Note that Eq. (12) is applicable when \( p \) is larger than \( D \). If \( p \) is smaller than \( D \), the

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following equation should be used.

\[ F = \phi [EC/(t + EC)] I_0 [1 - 10^{-(t + EC)/\beta}] \] \hspace{1cm} (13)

IRVING et al. (1987) have shown that the wavelength dependence of resting intrinsic absorbance can be well fitted by an empirical equation (Eq. (19) in their paper) for wavelengths greater than 420 nm. If it is assumed that their empirical equation can be extended to the UV region, using their mean values of constants (Table III of their paper), \( t \) is calculated to be about 8.25 cm\(^{-1}\) for 340 nm and 6.26 cm\(^{-1}\) for 380 nm. In this case, if the average path length of the muscle fiber is 157 \( \mu \)m, \( R_{\text{max}} \) is reduced by 6.1% when injected fura-2 concentration (\( C \)) is 100 \( \mu \)M, and by 20.2% when \( C \) is 400 \( \mu \)M, compared to \( R_{\text{max}} \) obtained from the in vitro calibration (fura-2 concentration, 50 \( \mu \)M) (Eq. (12)).