Resting Cytoplasmic Free Ca\(^{2+}\) Concentration in Frog Skeletal Muscle Measured with Fura-2 Conjugated to High Molecular Weight Dextran

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ABSTRACT Intact frog skeletal muscle fibers were injected with the Ca\(^{2+}\) indicator fura-2 conjugated to high molecular weight dextran (fura dextran, MW ~10,000; dissociation constant for Ca\(^{2+}\), 0.52 \(\mu\)M), and the fluorescence was measured from cytoplasm (17°C). The fluorescence excitation spectrum of fura dextran measured in resting fibers was slightly red-shifted compared with the spectrum of the Ca\(^{2+}\)-free indicator in buffer solutions. A simple comparison of the spectra in the cytoplasm and the in vitro solutions indicates an apparently "negative" cytoplasmic [Ca\(^{2+}\)], which probably reflects an alteration of the indicator properties in the cytoplasm. To calibrate the indicator's fluorescence signal in terms of cytoplasmic [Ca\(^{2+}\)], we applied β-escin to permeabilize the cell membrane of the fibers injected with fura dextran. After treatment with 5 \(\mu\)M β-escin for 30–35 min, the cell membrane was permeable to small molecules (e.g., Ca\(^{2+}\), ATP), whereas the 10-kD fura dextran only slowly leaked out of the fiber. It was thus possible to estimate calibration parameters in the indicator fluorescence in the fibers by changing the bathing solution [Ca\(^{2+}\)] to various levels; the average values for the fraction of Ca\(^{2+}\)-bound indicator in the resting fibers and the dissociation constant for Ca\(^{2+}\) (\(K_\text{D}\)) were, respectively, 0.052 and 1.0 \(\mu\)M. For the comparison, the \(K_\text{D}\) value was also estimated by a kinetic analysis of the indicator fluorescence change after an action potential stimulation in intact muscle fibers, and the average value was 2.5 \(\mu\)M. From these values estimated in the fibers, resting cytoplasmic [Ca\(^{2+}\)] in frog skeletal muscle fibers was calculated to be 0.06–0.14 \(\mu\)M. The range lies between the high estimates from other tetracarboxylate indicators (0.1–0.3 \(\mu\)M; Kurebayashi, N., A. B. Harkins, and S. M. Baylor. 1993. Biophysical Journal. 64:1934–1960; Harkins, A. B., N. Kurebayashi, and S. M. Baylor. 1993. Biophysical Journal. 65:865–881) and the low estimate from the simultaneous use of aequorin and Ca\(^{2+}\)-sensitive microelectrodes (<0.04–0.06 \(\mu\)M; Blatter, L. A., and J. R. Blinks. 1991. Journal of General Physiology. 98:1141–1160) recently reported for resting cytoplasmic [Ca\(^{2+}\)] in frog muscle fibers.

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INTRODUCTION

Because muscle contraction-relaxation is regulated by cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],), a change in the [Ca\(^{2+}\)] (\(\Delta[\text{Ca}^{2+}]\)) in response to depolarization of the transverse-tubular membrane has been extensively studied with a variety of methods. In addition to the importance of the \(\Delta[\text{Ca}^{2+}]\), the level of resting [Ca\(^{2+}\)], before activation may also strongly influence muscle function. With an elevated level of resting [Ca\(^{2+}\)], (a) the amount of Ca\(^{2+}\) stored inside the sarcoplasmic reticulum (SR) will likely increase; (b) the rate of Ca\(^{2+}\) release induced by subsequent depolarization might be decreased (Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) release; Schneider and Simon, 1988); and (c) the intracellular Ca\(^{2+}\) buffering power is expected to be altered because of the partial saturation of cytoplasmic Ca\(^{2+}\) binding sites (troponin, parvalbumin, etc.).

In amphibian skeletal muscle fibers, a cell type in which [Ca\(^{2+}\)], has been studied most extensively, the quantification of resting [Ca\(^{2+}\)], has been attempted with various methods, including Ca\(^{2+}\)-sensitive microelectrodes (Tsien and Rink, 1980; Coray, Fry, Hess, McGuigan, and Weingart, 1980; Lopez, Alamo, Caputo, Dipolo, and Vergara, 1983; Weingart and Hess, 1984; Blatter and Blinks, 1991); aequorin (Blakes, Wier, and Snowdowne, 1980; Konishi and Kurihara, 1987; Blatter and Blinks, 1991); and tetracarboxylate indicators such as fura-2 (Klein, Simon, Szucs, and Schneider, 1988; Suda and Kurihara, 1991), fura red (Kurebayashi, Harkins and Baylor, 1993), and fluo-3 (Harkins, Kurebayashi, and Baylor, 1993). However, methodological difficulties encountered in the above studies make the quantification of [Ca\(^{2+}\)], somewhat uncertain, and consequently the estimated values for the resting [Ca\(^{2+}\)], vary widely. For example, the recent careful studies with the tetracarboxylate indicators (Harkins et al., 1993; Kurebayashi et al., 1993) suggest that [Ca\(^{2+}\)], at rest is at least 0.1 \(\mu\)M and possibly as large as 0.3 \(\mu\)M. On the other hand, another very well controlled study, which used both Ca\(^{2+}\)-sensitive microelectrodes and aequorin, reports that resting [Ca\(^{2+}\)], is below the detection limit of their methods, and gives [Ca\(^{2+}\)], values of 0.04–0.06 \(\mu\)M as an upper limit. Major problems related to the use of these indicators appear to be the binding of the indicators to intracellular constituents, which alters Ca\(^{2+}\) affinity, the optical signals of the indicators, or both (e.g., Konishi, Olson, Hollingworth, and Baylor, 1988; Blatter and Blinks, 1991; Baker, Brandes, Schreur, Camacho, and Weiner, 1994).

In the present study, we used fura dextran, fura-2 conjugated to dextran (MW \(\sim 10,000\)), to study [Ca\(^{2+}\)], in resting frog skeletal muscle fibers. In vitro measurements indicated that fura dextran retained the spectral properties of fura-2, although fura dextran had a slightly lower affinity for Ca\(^{2+}\). To take advantage of the large molecular size of fura dextran, the cell membrane of fibers injected with fura dextran was treated with a low concentration of \(\beta\)-escin (Kobayashi, Kitazawa, Somlyo, and Somlyo, 1989), a saponin ester previously shown to permeabilize the cell membrane of frog skeletal muscle to relatively small molecules (e.g., ATP) while permitting a much slower leakage of intracellular macromolecules, such as fura dextran and proteins (Konishi and Watanabe, 1995). We found that the use of \(\beta\)-escin, in combination with the high molecular weight indicator, permitted calibration of the indicator fluorescence in the fiber interior in the presence of a major fraction of cellular macromolecules.
Some of the results have been presented previously in abstract form (Konishi and Watanabe, 1994a, b).

**METHODS**

Single muscle fibers dissected from a leg muscle (m. tibialis anterior) of frogs (*Rana temporaria*) kept at 10-12°C were used. A single fiber was mounted in the narrow trough (3 mm in width, 3 mm in depth, and 50 mm in length) of the experimental chamber placed on the stage of an inverted microscope for the optical measurements. The fiber was stretched to a sarcomere length of 2.7-2.8 μm between a fixed hook and the arm of a force transducer (BG-10, Kulite Semiconductor Products, Inc., Leonia, NJ), and fura dextran (fura-2 conjugated to dextran) was pressure-injected into cytoplasm from the microelectrode tip. Resting fluorescence measurements were also carried out at the sarcomere length 2.7-2.8 μm. However, for the fluorescence signals measured during muscle activity, the fiber was further stretched to a sarcomere length of 3.6-3.8 μm to minimize artifacts from fiber movement. Muscle fibers were electrically stimulated by a 500-μs pulse of 1.5× threshold through a pair of platinum-black plates running parallel to the fiber.

**Solutions and Chemicals**

The fibers were perfused with a continuous flow of normal Ringer’s solution containing (millimolar): 115 NaCl; 2.5 KCl; 1.8 CaCl₂; and 5 MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.10-7.15 (17°C). In the experiments with β-escin treatment, the fiber was briefly incubated in a Ca²⁺-free Ringer’s solution (1.8 mM CaCl₂ of normal Ringer’s solution was substituted with 2.0 mM MgCl₂ and 1.0 mM EGTA (ethylene glycol-bis(β-amino-ethyl ether)N,N,N',N'-tetraacetic acid)). The solution inflow was then turned off, and the following solution change was performed by four or five manual pipettings and suctions, each of which exchanged the volume equivalent to that of the trough (~0.5 ml). The temperature of the solution in the experimental chamber was maintained at 17-19°C by means of a Peltier cooling device. The compositions of the solutions for the β-escin treatment and the subsequent calibration are listed in Table I. All the solutions had in common 1.0 mM free Mg²⁺ ([Mg²⁺]), ionic strength 0.15 M, and pH 7.00 (±0.01 by KOH). Solutions of various free Ca²⁺ concentrations ([Ca²⁺]) were prepared by mixing a solution containing EGTA with no Ca²⁺ and a solution containing EGTA plus Ca²⁺ (e.g., solutions 7 and 8 of Table I) (cf., Horiiuti, 1988), and the [Ca²⁺] was calculated by solving the set of simultaneous equations using the equilibrium constants from the literature (Martell and Smith, 1974). The apparent dissociation constant thus assumed for the Ca²⁺-EGTA reaction for the experimental condition (ionic strength 0.15 M, pH 7.0) was 10⁻⁶.₉ M (= 0.41 μM). β-escin was obtained from Sigma Chemical Co. (St. Louis, MO; lot 107F05191). The single lot of fura dextran (potassium salt; 1.0 dye/molecule; MW ~10,000) was purchased from Molecular Probes, Inc. (Eugene, OR; lot 2921) and used throughout the study. The thin layer chromatogram of three fura dextran vials (on silica with butanol/acetic acid/water 4:1:1) revealed a broad fluorescent band of fura dextran with a very slight fluorescence of free fura-2. The result is consistent with the broad distribution of dextran molecular weight, as suggested by the manufacturer, and a minor contamination of free fura-2. All other chemicals, including EGTA (Nakalai Tesque Inc, Kyoto, Japan), Na₂ATP (Boehringer Mannheim GmbH, Mannheim, Germany), and CaCl₂ (1 M solution; BDH Chemicals Ltd., Poole, UK), were of the highest analytical grade. TBQ (2,5-Di-4-tert-butylhydroquinone, Tokyo Kasei Organic Chemicals, Tokyo, Japan) was dissolved in the Ringer’s solution with 0.01% DMSO as a solvent.

**Optical Instruments**

The details of the optical apparatus have been described elsewhere (Konishi, Suda, and Kurihara, 1993). An inverted microscope (Diaphot, Nikon, Tokyo, Japan) was equipped with a dual wavelength fluorometer (CAM 230, JASCO, Tokyo, Japan) that could alternately switch between two
excitation wavelengths (switching frequency 100 Hz–1 kHz) from two independent monochromators. For the excitation of fluorescence, either two light beams of different wavelengths were switched (dual-wavelength excitation) or single light beam was continuously illuminated (single-wavelength excitation). The excitation light beam had a halfwidth of 5 nm and was focused on the muscle fiber on the microscope stage with a UV-transmissive objective (20× CF Fluor 20, Nikon), and the emitted fluorescence at 500 nm (±20 nm) was collected through an interference filter (Corion Co., Holliston, MA) from the 800-μm diameter field of the objective. The output current of a photomultiplier tube (R268, Hamamatsu Photonics, Shizuoka, Japan) was converted to voltage, low-pass filtered at 10 Hz, and digitized at 20 Hz with 12-bit resolution. For the measurements on a millisecond time scale (see Figs. 2, 3, and 8), data were filtered at 1 kHz and digitized at 2–3 kHz.

**TABLE I**

| Solution no. | KMS | EGTA | CaMS₂ | Na₂ ATP | Na₂ CP | MgMS₂ | PIPES |
|--------------|-----|------|-------|---------|--------|--------|-------|
| **A 2 mM EGTA solutions** |     |      |       |         |        |        |       |
| 1. Relaxing  | 97  | 2.0  | 0     | 4.46    | 0      | 4.56   | 10.0  |
| 2. Rigor    | 103 | 2.0  | 0     | 0       | 0      | 1.06   | 20.0  |
| 3. Ca rigor  | 99  | 2.0  | 2.0   | 0       | 0      | 1.00   | 20.0  |
| **B 10 mM EGTA solutions** |     |      |       |         |        |        |       |
| 4. Relaxing  | 73  | 10.0 | 0     | 4.43    | 0      | 4.78   | 10.0  |
| 5. Relaxing/CP | 44  | 10.0 | 0     | 4.39    | 10.0   | 5.16   | 10.0  |
| 6. Activating/CP | 24  | 10.0 | 10.0  | 4.48    | 10.0   | 4.89   | 10.0  |
| 7. Rigor    | 79  | 10.0 | 0     | 0       | 0      | 1.28   | 20.0  |
| 8. Ca rigor  | 59  | 10.0 | 10.0  | 0       | 0      | 1.00   | 20.0  |

For all solutions, the pH was adjusted to 7.00 by adding KOH, the free Mg²⁺ concentration was calculated as 1.0 mM, and the ionic strength was 0.15 M. For solutions containing ATP (solutions 1, 4, 5, and 6), MgATP concentration was kept constant at 3.5 mM.

MS, methanesulfonate; CP, creatine phosphate; PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid].

Two types of fluorescence signals were measured.

**Fluorescence excitation spectrum.** Excitation wavelength, in the single-wavelength excitation mode, was changed from 330 to 420 nm with a step of 5 nm (10 nm for wavelengths >400 nm).

**Fluorescence ratio.** In most experiments, the ratio of the fluorescence intensity at two excitation wavelengths was measured (dual-wavelength excitation mode) instead of the more time-consuming measurement of the full spectrum. The switching frequency was 100 Hz for the measurements in the resting state and 1 kHz for the measurements during the muscle activity. To minimize the UV exposure, a pulse-controlled shutter for the excitation light beam was opened only when the fluorescence was measured. For the resting state fluorescence measurements, the fluorescence signals were averaged over a 2-s period.

In some experiments, we measured the fiber’s intrinsic absorbance, as described previously (Konishi et al., 1993); the field of illumination was restricted to a rectangular window on the muscle fiber, and the light transmitted through the fiber was detected by a photodiode detector set above the muscle fiber in the path of the incident light beam (see Fig. 1 of Konishi et al., 1993).
In vitro Fluorescence Measurements

Thin wall quartz capillaries (Vitro Dynamics, Inc., Rockaway, NJ) filled with buffer solutions containing fura dextran (11–76 µM) were placed in the muscle chamber on the inverted microscope, and the fluorescence signals of Ca²⁺-free and Ca²⁺-bound fura dextran were measured with the same optical arrangement as used for the muscle measurements. The shape of the excitation spectrum was independent of the indicator concentrations in this range. The composition of the standard buffer solution was 122 mM KCl, 10 mM NaCl, 10 mM PIPES, 1 mM MgCl₂ plus either 5 mM EGTA (for 0 [Ca²⁺]) or 0.5 mM CaCl₂ (for saturating [Ca²⁺]) (pH 6.9 at 17°C). The Ca²⁺-dependent change of the fura dextran excitation spectrum was very similar to that of the fura-2 spectrum, namely, a large blue shift of the spectrum on Ca²⁺ binding, with an isosbestic wavelength at 360 nm (Grynkiewicz, Poenie, and Tsien, 1985). For the fluorescence ratio measurements, we therefore chose 382 nm (the wavelength at which the fluorescence change caused by Ca²⁺ binding is maximal) and 360 nm (the isosbestic wavelength for Ca²⁺), and the ratio of the fluorescence intensities excited at 382 nm [F(382)] at 360 nm [F(360)] was calibrated in [Ca²⁺] unit [R = F(382)/F(360)]. The value of R in the Ca²⁺-free standard buffer solution was 1.26 with single-wavelength excitation (see Fig. 1) and 1.03 with dual-wavelength excitation. The lower value obtained with dual-wavelength excitation mode is the result of the lower efficiency of the second monochromator. For simplicity, the values of R are therefore normalized to that measured in the Ca²⁺-free standard buffer solution taken with the identical optics. The normalized R decreases from 1.0 to 0.08–0.09 (on average, 0.084) on Ca²⁺ binding, independent of the excitation mode. The F(360) is Ca²⁺ independent and is proportional to the indicator concentration in the optical field.

Fura dextran’s dissociation constant (K₀) for Ca²⁺ was estimated in vitro by measuring the fluorescence in the calibration solutions with various [Ca²⁺]’s, which were prepared by mixing solutions 7 and 8 of Table I (18°C, 1 mM [Mg²⁺]). The values of R at eight different [Ca²⁺]’s were least squares fitted with a theoretical 1:1 binding curve (for the details of the analysis, see Konishi et al., 1988). One run made in quartz capillaries on the microscope with 20 µM indicator gave a best-fitted K₀ value of 0.50 µM (the left-most curve in Fig. 7 D). Two other runs with a spectrofluorometer (FP-770, JASCO) with 1 µM fura dextran gave K₀ values of 0.51 µM and 0.56 µM. The average value from these three runs, 0.52 (±0.03) µM, is 2–2.5 times larger than that of fura-2 at 1 mM [Mg²⁺] (e.g., Konishi et al., 1988). Inclusion of ATP and creatine phosphate in the calibration solutions (solutions 5 and 6 of Table I) does not appreciably change the K₀ of fura dextran. In one run in quartz capillaries with 25 µM fura dextran, the R values measured at five different [Ca²⁺]’s (0, 0.2, 0.5, 1.0, and 60 µM) were consistent with a K₀ of 0.54 µM.

In Vivo Measurements

All experiments began with the measurements of “background” fluorescence, i.e., fiber fluorescence in the absence of indicator. Buffer solution containing fura dextran (5 mM fura dextran, 100 mM KCl, 2 mM PIPES, pH 7.0) then was pressure injected into the cytoplasm. In most experiments, the amount of fura dextran injected was large enough (>30 µM) to permit the long-term fluorescence measurements with a single impalement. Only fibers that responded in an all-or-none fashion after injection were used in the experiments. Fiber condition was also checked by applying a 50 Hz stimulus for 1 s, and the tetanic tension with a sustained plateau was taken as an additional criterion of a healthy fiber. The spatially averaged fura dextran concentration in cytoplasm was estimated by a simple comparison of the F(360) measured in the muscle fibers and in quartz capillaries of the same diameter, as described previously (Konishi et al., 1993; also Klein et al., 1988). The indicator concentration may be somewhat overestimated, as a quantum efficiency of the indicator molecules is likely to be increased in the cytoplasm from that in salt solutions (Konishi et al., 1988). The cytoplasmic fura dextran concentration, thus estimated, was generally 30–100 µM.
Immediately after the injection, fura dextran fluorescence was localized around the site of injection. The first fluorescence measurements were therefore carried out after a resting period of at least 30 min, during which fura dextran slowly diffused along the fiber axis. For the measurements of the indicator's excitation spectrum, \( F(360) \) was measured four times during the run to correct for a small time-dependent change in the indicator concentration, which is mostly caused by the diffusion of the indicator away from the injection site.

During the experiments, the background fluorescence (cell autofluorescence plus stray fluorescence) was also occasionally measured at a location far from the injection site (~2-3 mm), where the indicator concentration was negligible. When the fiber was perfused with Ringer's solution, the background fluorescence was essentially unchanged even during the longest experiments (~5 h). However, we found that the cell autofluorescence was somewhat changed in \( \beta \)-escin-treated fibers, particularly in rigor conditions (see below). In the experiments of \( \beta \)-escin-permeabilized fibers, we therefore subtracted off the background fluorescence measured close in time at the distant location to calculate the indicator fluorescence.

**Calibration of Fluorescence Signals**

If two populations of fura dextran molecules are assumed in the cytoplasm (a Ca\(^{2+}\)-free form and a Ca\(^{2+}\)-bound form), the fluorescence ratio \( R \) is a linear function of \( f_{\text{CaD}} \), the Ca\(^{2+}\)-bound fraction of the indicator, which lies between 0 (Ca\(^{2+}\)-free) and 1 (Ca\(^{2+}\)-bound):

\[
R = R_0 - f_{\text{CaD}} (R_1 - R_0),
\]

where \( f_0 \) and \( f_1 \) denote the fluorescence ratio of the Ca\(^{2+}\)-free \( (f_{\text{CaD}} = 0) \) and Ca\(^{2+}\)-bound \( (f_{\text{CaD}} = 1) \) indicator molecules, respectively. \( f_{\text{CaD}} \) can then be calibrated in terms of [Ca\(^{2+}\)] with the usual equation:

\[
[\text{Ca}^{2+}] = K_D \frac{f_{\text{CaD}}}{1 - f_{\text{CaD}}},
\]

where \( K_D \) denotes the dissociation constant of fura dextran for Ca\(^{2+}\).

**Statistical Tests**

Statistical values are given as means ± standard deviations. For statistical significance, the unpaired two-tailed Student’s \( t \) test was used with the significant level at \( P < 0.05 \), unless otherwise stated.

**RESULTS**

**Characterization of Fura Dextran Fluorescence Signals in Intact Muscle Fibers**

**Fura dextran fluorescence spectrum from resting fibers.** The fura dextran fluorescence excitation spectrum was measured from resting muscle fibers and compared with spectra obtained in the standard buffer solutions (Fig. 1). The fluorescence intensities measured at various wavelengths before the indicator injection (Fig. 1 A, solid circles) were subtracted from those measured after the injection (Fig. 1 A, open circles) to calculate the indicator-related spectrum (Fig. 1 B, open circles). The indicator fluorescence spectrum from the fiber was slightly red-shifted compared with the in vitro spectrum at 0 [Ca\(^{2+}\)] (0Ca in Fig. 1 B). Since Ca\(^{2+}\) binding causes a blue shift of the spectrum, the simple comparison of the spectra in the cytoplasm and the in vitro solution indicates an apparently “negative” [Ca\(^{2+}\)]. Indeed, a best fit of the
FIGURE 1. Comparison of the fluorescence excitation spectra of fura dextran measured in a muscle fiber and in the standard buffer solutions. Single-wavelength excitation mode; emission wavelength 500 ± 20 nm. (A) The "total" excitation spectrum obtained from a muscle fiber injected with fura dextran (open circles) and the "background" spectrum measured in the same portion of the muscle fiber just before the injection (solid circles). Fiber 07049292; estimated indicator concentration, 37-36 μM for the "total" excitation spectrum measured 88-100 min after injection. (B) (Open circles) The excitation spectrum of fura dextran in the muscle fiber obtained by the subtraction of the "background" spectrum (solid circles in A) from the "total" spectrum (open circles in A). The resulting spectrum was corrected for the small time-dependent change in the indicator concentration by linear interpolation between two adjacent measurements of F(360) and was normalized to average F(360). Dotted lines are in vitro spectra of 26 μM fura dextran obtained in Ca²⁺-free (0Ca) and 0.5 mM [Ca²⁺] (Sat. Ca) solutions in quartz capillaries (i.d., ~150 μM) placed in the muscle chamber. (C) (Crosses) The excitation spectrum obtained from the spectrum shown in B (open circles) after correction for the fiber's intrinsic absorbance. Dotted lines are the same in vitro spectra as shown in B. (D) (Crosses) The same excitation spectrum corrected for the fiber's absorbance as shown in C. Dotted lines were obtained in the buffer solutions with increased viscosity (2.1 cP) by addition of 630 mM sucrose. For each in vitro spectrum, data points measured at 5-10 nm intervals (small solid circles) were interpolated by means of a cubic spline algorithm (dotted line).

spectrum in the cytoplasm with a linear combination of the two in vitro spectra ("0Ca" and "Sat. Ca") yielded fCa₀ = -0.050. Very similar results were obtained in four other muscle fibers (fCa₀ = -0.048 ± 0.009, n = 5). In 48 resting fibers in which only the ratio signal was measured (see Methods), the normal value of R was
1.049 ± 0.018 (range, 1.010–1.089) in the cytoplasm. The $f_{calc}$ values calculated by Eq. 1 were $-0.053 ± 0.020$. A similar red shift of the excitation spectrum (not shown) was also observed in fibers injected with fura-2 (instead of fura dextran), which is consistent with that reported in cut fibers (Klein et al., 1988) and in intact fibers (Suda and Kurihara, 1991).

Since factors besides $[Ca^{2+}]_i$ must contribute to the indicator’s fluorescence signal in the cell, we carried out experiments aimed at assessing the contribution of several possible factors. We first considered the fiber’s intrinsic absorbance. At shorter wavelengths, the fiber’s absorbance is larger, so some fraction of excitation light should be absorbed by the fibers (Baylor, Chandler, and Marshall, 1982; Irving, Maylie, Sizto, and Chandler, 1987). For this purpose, the light intensity transmitted through muscle fibers was measured before the injection of fura dextran, and the intrinsic absorbance of the fiber was calculated, as described previously (see Fig. 3 of Konishi et al., 1993). The $A(\lambda)$ were then used for the correction of the fluorescence spectrum, as previously described (Eqs. 3 and 4 of Konishi et al., 1993). Fig. 1 C shows the fluorescence spectrum thus corrected for the fiber’s absorbance (crosses). After intrinsic absorbance correction, the muscle spectrum (crosses) was slightly closer to the in vitro spectrum at 0 $[Ca^{2+}]$, but still clearly red-shifted; the calculated $f_{calc}$ values were $-0.030 ± 0.010$ (five fibers).

We then considered cytoplasmic viscosity as the second possibility, because the myoplasmic viscosity is probably higher than that of water by a factor of about 2 (Kushmerick and Podolsky, 1969), and elevated viscosity was shown to affect the properties of fura-2 (Konishi et al., 1988; Poenie, 1990). The in vitro spectra of fura dextran measured in the standard buffer solutions at a viscosity of 2.1 cP (rather than 1.1 cP) are shown in Fig. 1 D. The fluorescence spectrum in the cytoplasm (corrected for the fiber absorbance; crosses) was still, but only slightly, red-shifted compared with the in vitro spectrum at 0 $[Ca^{2+}]$; the $f_{calc}$ values were estimated to be $-0.023 ± 0.017$ ($n = 5$). The important conclusion of the comparison shown in Fig. 1 is that the apparently “negative” cytoplasmic $[Ca^{2+}]$ cannot be entirely explained by the effects of the fiber intrinsic absorbance and cytoplasmic viscosity on the fluorescence of fura dextran, although these factors appeared to somewhat modify the fluorescence signals. We have further tested other possibilities that the cytoplasmic constituents might change the fluorescence signals. The fluorescence ratio $R$ was measured in vitro at 0 $[Ca^{2+}]$ (and, in some cases, also at saturating $[Ca^{2+}]$) by changing one of the following solution conditions: (a) the solution pH (pH of the standard buffer solution was changed between 6.8 and 7.2 either at 0 $[Ca^{2+}]$ or saturating $[Ca^{2+}]$); (b) solution ionic strength (ionic strength was increased from 0.15 M to 0.17 M by adding 20 mM KCl to the Ca$^{2+}$-free standard buffer solution); (c) choice of primary anion of the solution (KMS of solution 4 of Table I was replaced by equimolar of KCl or K glutamate, as glutamate is the most abundant amino acid in muscle fibers); (d) choice of primary cation (KMS of solution 7 of Table I was replaced by NaMS); (e) creatine phosphate and/or ATP either at 0 $[Ca^{2+}]$ (solutions 4, 5, and 7 of Table I) or saturating $[Ca^{2+}]$ (solutions 6 and 8 of Table I); and (f) carnosine (10 mM carnosine was added to solution 7 of Table I). None of these alterations caused detectable changes (>0.01) in the fluorescence ratio signal. An increase in $[Mg^{2+}]$ from 0 to 2 mM (at 0 $[Ca^{2+}]$) caused a
marginal decrease in $R (-0.017)$. In contrast to the minor effects, a small amount of albumin showed a profound effect; addition of bovine serum albumin (10 mg/ml) to the 0 [Ca$^{2+}$] solution (solution 4 of Table I) increased $R$ by 0.105. This finding led us to assume that cytoplasmic proteins, rather than small molecules, might primarily alter the indicator fluorescence, probably by binding indicator molecules as previously reported for fura-2 (Konishi et al., 1988).

Changes in fura dextran fluorescence during activity. Changes in fura dextran fluorescence in response to action potential stimulation ($\Delta F$) were measured at various

![Figure 2](image-url)

**Figure 2.** (A) Fura dextran fluorescence signals in response to an action potential stimulation (at time 0) at indicated excitation wavelengths (nm) near the trace $\Delta F(\lambda)$, in arbitrary unit. Each trace shown is a record from a single sweep, except for the trace at 360 nm (average of 7 sweeps). The traces were taken close in time but have been corrected for the time-dependent change in the indicator concentration by linear interpolation of the two bracketed $F(360)$ measurements. Single-wavelength excitation mode; emission 500 ± 20 nm. Fiber 071592fl, sarcomere length 5.7 μm; indicator concentration 31-29 μM; stimulation frequency 1/min. (B) Comparison of the wavelength dependence of the amplitude of peak $\Delta F(\lambda)$ obtained from the experiments of the type shown in A (open circles and crosses) and Ca$^{2+}$ difference spectra obtained in vitro with two different solution viscosities (1.1 cP, solid line; 2.1 cP, dotted line). Data from two muscle fibers were scaled to fit the average of the two in vitro spectra in the range 360-420 nm. (Open circles) fiber 071592fl, see above for other information. (Crosses) fiber 070492fl; sarcomere length 3.7 μm; 46-42 μM indicator. The in vitro spectra were measured at 5-10 nm intervals and were interpolated with the cubic spline algorithm for the purpose of graphic display. Only measured data points were used for the fit.

excitation wavelengths $\lambda$ (Fig. 2 A). Since $\Delta F(360)$ was very small and had the opposite polarity to $\Delta F(361)$, the isosbestic wavelength for Ca$^{2+}$ in cytoplasm is very close to 360 nm, which is the same isosbestic wavelength observed in vitro. The waveforms of the fluorescence change at shorter and longer excitation wavelengths were essentially identical; time to peak after stimulation and halfwidth were, respectively, 9.9 ± 0.6 ms and 30.4 ± 2.7 ms (6 fibers with the indicator concentration <50 μM). The time course of the fura dextran fluorescence signal was faster than
that of fura-2 (average time to peak and halfwidth, respectively, 14 and 55 ms; see also Baylor and Hollingworth, 1988), which is consistent with fura dextran's having a lower affinity for Ca\(^{2+}\) than fura-2 because of a larger dissociation rate constant for Ca\(^{2+}\).

The peak amplitude of \(\Delta F(\lambda)\) was plotted as a function of excitation wavelengths in Fig. 2 B (circles and crosses, data from two muscle fibers) and was compared with the Ca\(^{2+}\) difference spectra obtained under in vitro conditions either in 1.1 cP viscosity (the standard buffer solutions, solid line) or in 2.1 cP viscosity (sucrose added to the standard buffer solutions, dotted line). In spite of some uncertainties at wavelengths <360 nm because of cytoplasmic viscosity, the muscle \(\Delta F(\lambda)\) data were generally well matched to the in vitro Ca\(^{2+}\) difference spectra.

With one action potential stimulation, \(F(380)\) decreased, on average, by 64% from the resting level, (i.e., peak \(AR/R \sim -0.64\)). When the muscle was stimulated by a train of action potentials (100 Hz), \(\Delta R\) signals were summated (not shown) and peak \(\Delta R/R\) was -0.72 (average of two fibers). In an attempt to further drive intracellular indicator molecules into the Ca\(^{2+}\)-bound form, \([\text{Ca}^{2+}]\), was increased by application of 10 \(\mu\text{M}\) TBQ, an inhibitor of the SR Ca\(^{2+}\) pump (Westerblad and Allen, 1994a), in combination with high-frequency stimulation (67 Hz or 50 Hz). In one fiber stimulated at 67 Hz and a second fiber stimulated at 50 Hz in the presence of 10 \(\mu\text{M}\) TBQ, \(R\) reached 14.7% of the resting \(R\) measured before application of TBQ (i.e., peak \(AR/R\) was -0.853). This gives the upper limit of \(R\) in the cell of 0.15.

**Long-term stability of fura dextran fluorescence signals.** Since fura dextran molecules slowly diffuse away from the optical field during an experiment, we could examine the indicator concentration dependence of the fluorescence signal by simply following the \(R\) as a function of time after injection. In four muscle fibers which initially contained 42–108 \(\mu\text{M}\) fura dextran, the \(R\) values were occasionally monitored for 240–307 min after injection (cf. Fig. 3). Even though the indicator concentration estimated from \(F(360)\) decreased by \(\sim60\%\) by the end of the \(\sim5\text{-h}\) experiments, the resting \(R\) values were stable throughout the experiments. The slope of the least-squares-fitted line to the \(R\)versus-time plot in each fiber was very close to zero; the average slope was \(+0.10\% \pm 0.175\% \text{ h}^{-1}\), which was not significantly different from zero.

In some experiments, \(\Delta F(380)\) was occasionally monitored by application of stimuli (Fig. 3). The fractional change in \(F(380), \Delta F(380)/F(380)\), was taken to reflect \(\Delta[\text{Ca}^{2+}]\), (cf., right upper traces in Fig. 3). The amplitude and the waveform of the \(\Delta F(380)/F(380)\) signals were essentially unchanged, as indicated by the plot of the peak (Fig. 3 B) and the halfwidth (Fig. 3 C) vs time after injection, whereas the estimated indicator concentration decreased to less than half (Fig. 3 A). In three fibers, the slope of the least-squares-fitted lines were \(+0.24\% \pm 0.16\% \text{ h}^{-1}\) for the peak amplitude, and \(+1.07\% \pm 2.10\% \text{ h}^{-1}\) for the halfwidth, neither of which was significantly different from zero.

These experiments strongly suggest that, with the indicator concentration of \(\sim100 \mu\text{M}\) or lower, the fura dextran \(R\) signal was not influenced by the change in the indicator concentration (e.g., as a result of the inner filter effect), and that the \([\text{Ca}^{2+}]\), at rest and during activity was not altered by the Ca\(^{2+}\) buffering of the indicator.
Figure 3. Long-term stability of fura dextran fluorescence signals during activity. (A–C) Three types of data from two muscle fibers were plotted as a function of time after indicator injection. (Open circles) Fiber 041793fl; sarcomere length 3.6 μm. (Crosse) Fiber 033093fl; sarcomere length 3.6 μm. (A) Spatially averaged cytoplasmic concentration of fura dextran in the optical field. (B) The peak amplitude of the fractional fluorescence change at 380-nm excitation (ΔF/F, the fluorescence change normalized to the resting level) in response to single action potential. (C) The half-width of the ΔF/F at 380 nm. Single-wavelength excitation mode; emission wavelength 500 ± 20 nm. Upper right panel shows the examples of ΔF/F signals at 380 nm stimulated (at time 0) by a single stimulus. The four traces (a–d) were taken in fiber 033093fl at the times indicated in A.

Fluorescence Measurements in Permeabilized Muscle Fibers

Permeabilization of cell membrane by β-escin. To calibrate the indicator signals in terms of [Ca^{2+}], by means of Eqs. 1 and 2, the measurement of R in the cytoplasm and the estimates of three calibration parameters (R_i, R_0, and K_0) have to be obtained. Since one or more of these calibration parameters is likely to be altered in the cytoplasm (for example, because of the indicator binding to proteins), it was important to estimate the calibration parameters in the cytoplasm (in vivo calibration). One way to do this is to permeabilize the cell membrane selectivity to small molecules, while retaining cellular macromolecules so that the [Ca^{2+}], can be controlled (Williams and Fay, 1990). We have tested two Ca^{2+} ionophores, Br-A23187 (Sigma Chemical Co.) and ionomycin (Sigma Chemical Co.). Four fibers treated with Br-A23187 (10–200 μM) and two fibers treated with ionomycin (50–100 μM) devel-
oped strong irreversible contracture even after ~30 min of incubation in the 10 mM EGTA solution (solution 4 in Table I), which eventually destroyed the fiber structure. The contracture caused by the Ca\textsuperscript{2+} ionophores is probably caused by a leakage of Ca\textsuperscript{2+} from the SR, because these ionophores are known to permeabilize the SR membrane at low concentrations (Itoh, Kanamura, and Kuriyama, 1985). We therefore conclude that the Ca\textsuperscript{2+} ionophores may be difficult to use in frog skeletal muscle fibers, the SR of which is not easily depleted even after long-term incubation in the Ca\textsuperscript{2+}-free condition (Luttgau and Spiecker, 1979). In smooth muscle cells, β-escin, a saponin ester, has been shown to permeabilize the cell membrane to lower molecular weight proteins (e.g., calmodulin), whereas activity of the SR is retained (Kobayashi et al., 1989). In frog skeletal muscle fibers, we have previously shown that a low concentration of β-escin (5 μM) rendered the cell membrane permeable to relatively small molecules (e.g., ATP), without a substantial loss of large cytoplasmic proteins (14–80 kDa) (Konishi and Watanabe, 1995). It has been also shown that 10 kDa fura dextran only slowly leaked out of the fiber permeabilized with 5 μM β-escin. We therefore used β-escin to calibrate the fura dextran fluorescence signal in the presence of cytoplasmic proteins.

**General observations of β-escin treated fibers.** When the fiber was treated with 5 μM β-escin in the relaxing solution (solutions 1 or 4 of Table I), the fiber diameter measured under the microscope increased by 16% ± 9% after ~20 min (18 muscle fibers). This swelling of the fiber, which probably reflects the increase of the myofilament spacing as observed in mechanically skinned fibers or saponin-treated fibers (Matsubara and Elliott, 1972; Endo and Iino, 1980), can be taken as an indication that intracellular small molecules are well exchanged with those in the bathing solution. The muscle fiber structure appeared to be well retained, as judged from the clear striations observed under the microscope and the presence of sharp laser diffraction lines (not shown). The fiber autofluorescence measured in the absence of indicator did not change significantly; in 16 fibers treated with 5 μM β-escin for 30 min, the autofluorescences at 360-nm excitation and 382-nm excitation were, respectively, 92% ± 20% and 128% ± 36% of those measured in the Ringer’s solution. When ATP was removed from the bathing solution of β-escin treated fibers, however, a large decrease in the autofluorescence was observed; the autofluorescences at 360-nm excitation and 382-nm excitation were, respectively, 36% ± 19% and 62% ± 35% (n = 13) of those of the intact fibers.

**Estimation of R\textsubscript{0}.** Fig. 4 shows an example of an experiment in which the Ca\textsuperscript{2+}-independent F(360) of fura dextran, the Ca\textsuperscript{2+}-dependent R [F(382)/F(360)], and force were followed in the same fiber. Application of 5 μM β-escin in the relaxing solution (labeled "1 + β" in Fig. 4) caused two clear changes in the fura dextran fluorescence signals: (a) The acceleration of the decline in F(360) (Fig. 4, top) was consistent with cell membrane permeabilization and the resultant slow leak of 10-kD fura dextran molecules from the fiber; (b) onset of a small (~5%) but steady increase in R (Fig. 4, middle), as expected for the leak of intracellular Ca\textsuperscript{2+} out of the cell into the bathing solution, which contained a high concentration (2 mM) of EGTA. Since the volume of the bathing solution was ~1,000 times larger than the cell volume, EGTA in the bathing solution should effectively bind most of the Ca\textsuperscript{2+} that leaked from the cell. In addition, extracellular EGTA (MW 380) probably
could enter the cell to directly buffer Ca\(^{2+}\) in the cell interior, because the cell membrane treated with 5 \(\mu\)M \(\beta\)-escin is permeable to ATP (MW \(\sim\)580) (Konishi and Watanabe, 1995; also see below).

Because fura dextran slowly leaked out of the fiber through the permeabilized cell membrane, a part of the fluorescence might be emitted from the indicator molecules in the extracellular space. To check this possibility, the extracellular space was occasionally flushed with fresh solution just before the fluorescence measurements, as indicated by the arrows in Figs. 4, 6, and 7. Neither the \(R\) nor the decay rate of \(F(360)\) was noticeably affected by this procedure, suggesting that the fluorescence measurements are not affected by contaminating fluorescence from the extracellular space.

![Figure 4](image-url)

**Figure 4.** The fluorescence signals of fura dextran (upper two panels) and force (bottom panel) followed for \(~\sim 200\) min after the indicator injection (at time 0 on the abscissa) in a muscle fiber. The top panel shows the \(F(360)\) signal, and the second panel shows the fluorescence ratio \(R\), \(F(382)/F(360)\). The force is expressed as a relative term to near maximal force measured during tetanus (50 Hz, \(1\ s\)) at the beginning of the experiment (in the Ringer's solution). Fiber 06123937; indicator concentration 97-9 \(\mu\)M. The fiber was initially incubated in the Ringer's solution; the bathing solution was sequentially changed, as indicated at the top of the figure: 1 + \(\beta\), relaxing solution (2 mM EGTA) plus 5 \(\mu\)M \(\beta\)-escin; 1, relaxing solution (2 mM EGTA); 2, rigor solution (2 mM EGTA); 3, Ca rigor solution (2 mM CaMS\(_2\) and 2 mM EGTA) plus 1 mM CaCl\(_2\) (pCa \(\sim\) 3). For details of the solution compositions, see Table I. Arrows indicate the flush of the bath by the fresh solution. Dual-wavelength excitation mode (switching frequency 100 Hz); emission wavelength 500 ± 20 nm.
Removal of ATP from the bathing solution (labeled 2 in Fig. 4), while keeping [Mg\(^{2+}\)] at 1 mM and [Ca\(^{2+}\)] low by 2 mM EGTA (solution 2 in Table I), caused force generation of the muscle fiber (Fig. 4, bottom). This force generation is consistent with the loss of intracellular ATP through the permeabilized cell membrane and subsequent formation of rigor cross-bridges, as the force was completely reversed by the re-addition of ATP in the bathing solution (see label 1 in Fig. 4). The unexpected result was, however, that “the Ca\(^{2+}\)-dependent signal,” R, also changed by this procedure; the removal of extracellular ATP caused an increase in R, which was reversible by reapplication of ATP and could be repeated many times (Fig. 4, middle). This change in R induced by removal of ATP was very similar to that observed in the solution containing 10 mM EGTA (solution 7 of Table I) in which Ca\(^{2+}\) is very heavily buffered (see Fig. 6 A). Since most of the ATP molecules in the solutions are in the Mg\(^{2+}\) bound form (MgATP), we measured R from the permeabilized fibers at five different [MgATP]'s (0, 1, 2, 3.5, and 5 mM), while keeping most of other solution constituents constant (10 mM EGTA, 1 mM free Mg\(^{2+}\), pH 7.0, ionic strength 0.15 M). We found that R was only very slightly affected by lowering the [MgATP] from 5 mM to 2 mM (+1.1% increase, average of two fibers), but steeply increased at a [MgATP] lower than 2 mM. Since cytoplasmic MgATP level in intact frog skeletal muscle fibers is thought to be in the several millimolar range (e.g., 5.7 mM; Godt and Maughan, 1988), we assumed that, for the estimation of R\(_0\) in the physiologic cytoplasm, the R value measured at 3.5 mM [MgATP] was probably a better estimate than that measured in the absence of MgATP. The fiber autofluorescence was also dramatically changed by the removal of ATP from the cytoplasm (see above).

To further check if the change in R after cell membrane permeabilization in the presence of 3.5 mM MgATP (see I+β time period in Fig. 4) truly reflects a decrease in [Ca\(^{2+}\)]\(_i\), we measured and compared the fluorescence spectra before and after cell membrane permeabilization. If the R change solely reflects a change in [Ca\(^{2+}\)]\(_i\), the wavelength dependence of the fluorescence change upon permeabilization should match the Ca\(^{2+}\) difference spectrum obtained in vitro (see Fig. 2 B). The results are summarized in Fig. 5. After the spectrum was measured in an intact fiber (Fig. 5 A, circles), the fiber was treated with 5 μM β-escin in the relaxing solution (solution 1 of Table I), and the spectrum was remeasured (Fig. 5 A, crosses). If the two spectra are normalized to each F(360) to correct for the decline of the indicator concentration (because of the diffusion plus the leak), the F(380) was slightly increased after the β-escin treatment, in agreement with the effect shown in Fig. 4. The subtraction of crosses from circles in Fig. 5 A showed that the fluorescence change upon cell membrane permeabilization had a similar wavelength dependence to the in vitro Ca\(^{2+}\) difference spectrum (Fig. 5 B).

For comparison, similar experiments were carried out with the rigor solution lacking ATP (solution 2 of Table I). The permeabilization by β-escin in the rigor solution caused a large increase in the F(380) normalized to F(360) (Fig. 5 C), which is again consistent with the results described above. However, the wavelength dependence of the fluorescence change was quite different from the in vitro Ca\(^{2+}\) difference spectrum (Fig. 5 D). The results suggest, in support of the above assumption, that the fluorescence change on permeabilization in the absence of ATP con-
FIGURE 5. Excitation wavelength dependence of the fura dextran fluorescence change by cell membrane permeabilization. Single-wavelength excitation mode, emission wavelength 500 ± 20 nm. (A) Excitation spectra of fura dextran measured in a fiber before β-escin treatment in the Ringer's solution (open circles) and after the cell membrane permeabilization in the relaxing solution (solution 1 of Table I; Crosses). Each spectrum has been corrected for the decrease in the indicator concentration during the measurements (see legend of Fig. 1) and normalized to the average value of F(360). Fiber 062993f1; indicator concentration, 56-53 μM for circles and 21-18 μM for crosses. (B) (Crosses) the difference spectrum calculated by subtraction of crosses from circles in A. (Triangles) data obtained from a similar experiment to that shown in A in a different muscle fiber (not shown). (C) Fura dextran spectra obtained, as described above, in a fiber before β-escin treatment in the Ringer's solution (open circles) and after the cell membrane permeabilization in the rigor solutions of very low [Ca²⁺] (solution 2 of Table I; Crosses) and ~1 mM [Ca²⁺] (1 mM CaCl₂ added to solution 3 of Table I; solid circles). Fiber 063093f1; indicator concentration, 79-75 μM for circles, 38-36 μM for crosses, 33-30 μM for solid circles. (D) The difference spectra calculated from the spectra shown in C. (Open circles) subtraction of crosses from open circles in C. (Solid circles) subtraction of crosses from closed circles in C. Open triangles and solid triangles were obtained in the same manner as open circles and solid circles, respectively, based on the similar measurements in a different muscle fiber (not shown). In A and C, dotted lines are in vitro spectra of ~50 μM fura dextran in the buffer solutions of very low [Ca²⁺] (labeled 0Ca, solution 2 of Table I) and saturating [Ca²⁺] (labeled Sat. Ca, 1 mM CaCl₂ added to solution 3 of Table I) filled in quartz capillaries (i.d., ~100 μM). For each 0Ca and Sat. Ca spectrum, the spectra measured in the 1.1 and 2.1 cP solutions at 5-nm intervals were averaged and interpolated with the cubic spline algorithm for the display. The Ca²⁺ difference spectrum calculated, point by point, by subtraction of the Sat. Ca spectrum from the 0Ca spectrum is shown in B and D. In B and D, each data set obtained in muscle fibers has been scaled to fit the in vitro difference spectrum in the range of 360-410 nm.
tains some component not related to [Ca²⁺], and that R measured in the relaxing solution gives a more reliable estimate of the R₀ in the intact fibers.

The R₀ values, thus estimated in the relaxing solution containing either 2 mM (A) and 10 mM EGTA (B), are summarized in column 4 of Table II. On average, R₀ values measured in 10 muscle fibers with 2 mM EGTA and those measured in 11 muscle fibers with 10 mM EGTA are not statistically different (non-paired t test). However, in four muscle fibers in which R₀ values in 2 mM EGTA and 10 mM EGTA were compared in the same fibers (070993f1, 070993f2, 071093f1, and 071093f2), R₀ values in 10 mM EGTA were slightly but significantly higher than those in 2 mM EGTA (0.01 < P < 0.05, paired t test).

In one muscle fiber in Table II (B) (indicated by an asterisk), 10 mM creatine phosphate was included in the bathing solution (solution 5 of Table I). Two other fibers (not included in Table II, because of a slight indication of the injection damage) gave R₀ values of 1.122 and 1.144 in the presence of 10 mM creatine phosphate. These values of R₀ (1.101–1.144) measured in the presence of 10 mM creatine phosphate were in the range of and not statistically different from the R₀ val-

![Figure 6](image-url)

**Figure 6.** The change in the fluorescence ratio R, F(382)/F(360), by Ca²⁺ of saturating concentrations. (A and B) The results from two separate experiments in which slightly different experimental protocols and solutions were employed (see text for details). (A) Fiber 110293f1, (B) fiber 051793f1. For A and B, the upper panels show the fluorescence intensity at 360-nm excitation, and the lower panels show the fluorescence ratio. The abscissa is the time after the indicator injection. The changes in the bathing solutions are indicated at the top of A and B: 4 + β, the relaxing solution (10 mM EGTA) plus 5 μM β-escin; 4, the relaxing solution (10 mM EGTA); 7, the rigor solution (10 mM EGTA); 8, Ca rigor solution (10 mM CaMS₂ and 10 mM EGTA) plus 1 mM CaCl₂ (pCa ~ 3); 2* + β, rigor solution (2 mM EGTA) plus 5 μM β-escin with replacement of KMS by NaMS; 2, the rigor solution (2 mM EGTA); 3, Ca rigor solution (2 mM CaMS₂ and 2 mM EGTA, pCa 4.5). For the compositions of the solutions, see Table I. Arrows indicate the flush of the bath by the fresh solution. Dual-wavelength excitation mode (switching frequency 100 Hz), emission wavelength 500 ± 20 nm.
TABLE II
Analysis of Fluorescence Measurements of Fura Dextran in Intact and Permeabilized Fibers

| Fiber no. | Fura dextran, µM | Intact | Permeabilized |  |  |  |
|-----------|----------------|--------|---------------|---|---|---|
| (1)       |                | (2)    | (3)           | (4) | (5) | (6) |
| A 2mM EGTA solutions |               |        |               |   |    |    |
| 060893f2  | 35-14          | 1.084  | 1.117 (0.096) | 0.0327 |       |   |
| 060993f1  | 118-22         | 1.053  | 1.087 (0.096) | 0.0550 |       |   |
| 061293f1  | 92-35          | 1.052  | 1.093 (0.096) | 0.0415 |       |   |
| 061493f1  | 73-28          | 1.034  | 1.101 (0.096) | 0.0573 |       |   |
| 061793f2  | 35-15          | 1.067  | 1.120 (0.096) | 0.0523 |       |   |
| 061993f2  | 41-10          | 1.077  | 1.093 (0.096) | 0.0162 |       |   |
| 070993f1  | 27-10          | 1.070  | 1.098 (0.096) | 0.0282 |       |   |
| 070993f2  | 66-13          | 1.055  | 1.080 (0.096) | 0.0256 |       |   |
| 071093f1  | 66-16          | 1.046  | 1.089 (0.096) | 0.0437 |       |   |
| 071093f2  | 27-7           | 1.039  | 1.071 (0.096) | 0.0331 |       |   |
| Mean ± SD |                | 1.056  | 1.095         | 0.040  | ±0.018 | ±0.015 | ±0.016 |
| B 10 mM EGTA solutions |               |        |               |   |    |    |
| 070993f1  | 27-10          | 1.070  | 1.112 (0.096) | 0.0418 |       |   |
| 070993f2  | 66-13          | 1.055  | 1.087 (0.096) | 0.0326 |       |   |
| 071093f1  | 66-16          | 1.056  | 1.109 (0.096) | 0.0628 |       |   |
| 071093f2  | 27-7           | 1.039  | 1.090 (0.096) | 0.0518 |       |   |
| 071293f1  | 143-18         | 1.032  | 1.108 (0.096) | 0.0759 |       |   |
| 10599f1   | 62-17          | 1.089  | 1.148 (0.100) | 0.0568 |       |   |
| 110293f1  | 37-12          | 1.062  | 1.100 (0.096) | 0.0382 |       |   |
| 110293f2  | 103-23         | 1.045  | 1.096 (0.120) | 0.0514 |       |   |
| 111793f1  | 129-44         | 1.052  | 1.105 (0.098) | 0.0532 |       |   |
| 111793f2  | 71-22          | 1.049  | 1.095 (0.094) | 0.0464 |       |   |
| 120693f1* | 108-48         | 1.043  | 1.101 (0.096) | 0.0583 |       |   |
| Mean ± SD |                | 1.053  | 1.105         | 0.052  | ±0.016 | ±0.016 | ±0.012 |

For each fiber (column 1), fura dextran fluorescence ratio signal (R, column 3) was first measured in Ringer's solution. Then the cell membrane was permeabilized with β-escin, and the fluorescence ratio at 0 Ca²⁺ (R₀, column 4) was measured in the relaxing solution, which contained 3.5 mM MgATP and buffered with either 2 mM (part A) or 10 mM (part B) EGTA. In one fiber with an asterisk, the relaxing solution also contained 10 mM creatine phosphate. The values listed in columns 3 and 4 are the average of several measurements. In five fibers, the Ca²⁺-dependent change in the fluorescence ratio (R₁/R₀, column 5) was also estimated in the same fibers in the rigor condition, as shown in Fig. 6A. In other fibers, the average value of R₁/R₀ (0.096) estimated in the separate experiments was used for the analysis, as indicated by parentheses in column 5. Column 2 gives the spatially averaged indicator concentration during the measurements in columns 3–5. Column 6 shows the estimates of fCaD calibrated from the fluorescence signals in columns 3–5.
Figure 7. The fluorescence ratio \( R, F(382)/F(360) \), of fura dextran measured in \( \beta \)-escin treated fibers at various bathing solution \([Ca^{2+}]\)'s. (A) An experiment with the solutions lacking ATP. After the permeabilized fiber was equilibrated in the rigor solution with 10 mM EGTA (marked EGTA; solution 7 in Table I), the bathing solution \([Ca^{2+}]\) was sequentially changed as indicated at the top (in pCa units). The solution of pCa 4.2 is the Ca rigor solution (10 mM CaMS² and 10 mM EGTA; solution 8 in Table I). (B) A similar experiment as shown in A, but bathing solutions contained 3.5 mM MgATP and 10 mM creatine phosphate. The relaxing solution (marked EGTA) contained 10 mM EGTA (solution 5 of Table I), and the bathing solution \([Ca^{2+}]\) was increased up to \( \sim 300 \) nM, as indicated (in pCa units) at the top. In A and B, the abscissa is the time after indicator injection, and arrows indicate the flush of the bath by the fresh solution. Dual-wavelength excitation mode (switching frequency 100 Hz); emission wavelength 500 ± 20 nm. (C) The analysis of the accumulated data from the type of experiments shown in A and B. Solid symbols, data from four experiments carried out in the rigor fibers as shown in A. Open symbols, data from three experiments carried out in the solutions containing 3.5 mM MgATP and 10 mM creatine phosphate as shown in B. The average \( R \) values of two to four measurements in each solution (as indicated by horizontal lines in A and B) are plotted versus pCa. Also shown (small crosses) are data obtained in buffer solutions in quartz capillaries (i.d., \( \sim 150 \) \( \mu \)m) placed in the muscle chamber. The solutions for the in vitro measurements were made by mixing solutions 7 and 8 (Table I) and contained 20 \( \mu \)M fura dextran. A solid line is a theoretical 1:1 binding curve of \( K_0 = 0.50 \) \( \mu \)M least-squares fitted to the in vitro data. (D) The muscle data in C are normalized to the value obtained in EGTA solutions without \( Ca^{2+} \) (either rigor or relaxing solution) in each fiber, and replotted versus pCa under the same symbols. Three solid lines are theoretical 1:1 binding curves with \( K_0 \)'s (left to right) 0.50 \( \mu \)M, 0.81 \( \mu \)M, and 1.14 \( \mu \)M, best-fitted to the crosses, solid symbols, and open symbols, respectively. The indicator concentrations during the measurements: solid circles (fiber 051893fl), 38-21 \( \mu \)M; solid triangles (fiber 051993fl), 47-20...
ues measured in the absence of creatine phosphate, suggesting that the estimate of the $R_0$ in the fibers is not critically influenced by intracellular creatine phosphate. For additional checks concerning influences of the bathing solution constituents on $R_0$, measurements were made from permeabilized fibers in the relaxing solution (solution 4 of Table I) with one of the following modifications: (a) a change in the solution pH from 7.0 to either 6.8 or 7.2 (2 fibers); (b) an increase in $[\text{Mg}^{2+}]$ from 1 mM to 2 mM, while keeping the MgATP concentration constant at 3.5 mM (2 fibers); and (c) addition of 10 mM carnosine (2 fibers); no substantial change in the $R_0$ (<0.01) was observed, as described above for the in vitro conditions.

Estimation of $R_1/R_0$. When the $[\text{Ca}^{2+}]$ in the rigor solution was raised to ~1 mM, the $R$ signal quickly decreased to a very low level (<0.2), as expected for the rise of $[\text{Ca}^{2+}]$, with no further increase in the rigor force (Fig. 4). The wavelength dependence of the fluorescence change on addition of $\text{Ca}^{2+}$ (Fig. 5D, solid symbols) was similar to the $\text{Ca}^{2+}$ difference spectrum measured in vitro (Fig. 5D, dotted line), suggesting that the fluorescence change was primarily caused by $\text{Ca}^{2+}$ binding.

Because high $[\text{Ca}^{2+}]$ in the presence of ATP causes a large contraction and accompanying fiber movement (which makes the reliable fluorescence measurements very difficult), we decided to estimate the relative change in $R$ induced by $\text{Ca}^{2+}$, $R_1/R_0$, in the absence of ATP, where there is little $\text{Ca}^{2+}$-activated force generation. Fig. 6 shows examples of the $R$ change in the saturating $[\text{Ca}^{2+}]$'s recorded in two experiments with slightly different protocols. In Fig. 6A, after the fiber was permeabilized by β-escin in relaxing solution (labeled 4 + β), β-escin was washed out (4), and ATP in the bathing solution was removed (7). In the experiment shown in Fig. 6B, the muscle fiber was treated with β-escin in the rigor solution (2 + β). In spite of the small differences in the protocol and the solution compositions (e.g., 10 mM EGTA in A, and 2 mM EGTA in B), a very similar $\text{Ca}^{2+}$-dependent change in the $R$ was observed; addition of $\text{Ca}^{2+}$ to the bathing solution caused a large decrease in $R$, by ~90%, which was completely reversed on removal of $\text{Ca}^{2+}$ from the extracellular space. In nine muscle fibers, the average value of $R_1/R_0$, thus estimated at ~1 mM bathing solution $[\text{Ca}^{2+}]$, was 0.096 (± 0.011). The reduction in the bathing solution $[\text{Ca}^{2+}]$ to 60 µM did not cause a significant change in the estimate of $R_1/R_0$ (0.103 ± 0.005, $n = 7$), consistent with the saturation of the intracellular indicator with $\text{Ca}^{2+}$.

Estimation of the dissociation constant. Because $\text{Ca}^{2+}$ appears to quickly diffuse across the permeabilized cell membrane, as shown in the previous section, the dissociation constant of fura dextran for $\text{Ca}^{2+}$ could be estimated by setting the bathing solution $[\text{Ca}^{2+}]$ to various levels. For this purpose, we selected relatively small muscle fibers (diameter 55–90 µm) to facilitate radial diffusion of $\text{Ca}^{2+}$, and 10 mM EGTA was used to ensure the stable buffering of $\text{Ca}^{2+}$ (solutions 5–8 of Table I). Fig. 7 shows results from two muscle fibers and analyzes the data from seven muscle fibers:

- Solid squares (fiber 042493f2), 46–27 µM;
- Solid diamonds (fiber 042793f1), 27–18 µM;
- Open circles (fiber 042093f1), 69–45 µM;
- Open triangles (fiber 120693f1), 50–37 µM;
- Open squares (fiber 120593f1), 61–40 µM.
fibers. In Fig. 7 A, the muscle fiber had been incubated in the rigor solution lacking ATP for ~20 min at time zero on the abscissa, and all solutions did not contain ATP (mixture of solutions 7 and 8) during the measurements, so the muscle tension was not increased above rigor level (not shown). The value of $R$ changed after the change in bathing solution $[\text{Ca}^{2+}]$, and reached new steady levels within a few minutes. Fig. 7 B shows the result of a similar experiment in which the bathing solutions contained ATP (and creatine phosphate) (mixtures of solutions 5 and 6 of Table I). In this experiment, the bathing solution $[\text{Ca}^{2+}]$ was not raised to higher than 0.3 μM to avoid $\text{Ca}^{2+}$-activated force generation and accompanied fiber movement. Figure 7 C summarizes the steady-state relationship between $[\text{Ca}^{2+}]$ and the $R$ signal estimated in these and five other fibers, under the assumption that $[\text{Ca}^{2+}]_i$ was in equilibrium with the bathing solution $[\text{Ca}^{2+}]$ (open and solid symbols). The analysis of the $\text{Ca}^{2+}$ dissociation constant of fura dextran is shown in Fig. 7 D. We separately analyzed two data sets; the data from the rigor fibers and those in the solutions containing ATP. The combined data obtained in the rigor fibers (solid symbols) were least-squares fitted with a 1:1 binding curve by adjusting two parameters, a dissociation constant and the value of $R_1/R_0$. The best-fitted value of the $K_D$ thus obtained was 0.81 μM, and the $R_1/R_0$ was 0.084, a value close to that estimated in the previous section (0.096). A very similar analysis was applied to the combined data obtained in the presence of ATP (and creatine phosphate) (open symbols), but the $R_1/R_0$ value was fixed to 0.096, the value estimated in the previous section. This was necessary to obtain a reliable fit, because the data cover only a part of the binding curve. The best-fitted $K_D$ value was 1.14 μM, somewhat higher than 0.81 μM estimated in the rigor fibers. We do not know which estimate of the two more closely reflects the $K_D$ in intact muscle fibers; the data from the rigor fibers cover the full range of $[\text{Ca}^{2+}]$, but could suffer from some artifact because of the lack of ATP; the data in the ATP-containing solutions might be physiologically more relevant, but the $[\text{Ca}^{2+}]$ range is limited. We therefore somewhat arbitrarily took the average of the two estimates, and obtained the value of 1.0 μM as an estimate of the in vivo dissociation constant. The value of 1.0 μM is larger than that obtained in vitro by a factor of about two.

Estimation of the resting $[\text{Ca}^{2+}]$. With the three parameters ($R_0$, $R_1/R_0$, and $K_D$) estimated in the β-escin–treated fibers, as described above, the resting $[\text{Ca}^{2+}]$ was calculated by means of Eqs. 1 and 2 for each experimental measurement of $R$ (Table II). Table II summarizes the results obtained from 17 muscle fibers that satisfied our criteria of healthy fibers (all-or-none response and complete tetanus tension). In each muscle fiber, $R$ was first measured in Ringer’s solution before β-escin treatment, and then $R_0$ was measured after the β-escin treatment in the relaxing solution containing either 2 mM (A) or 10 mM EGTA (B). In five experiments (fibers 10593f1, 11093f1, 11093f2, 111793f1, and 111793f2), the fibers were further transferred to the rigor solution, and the $R_1/R_0$ was estimated in the same fibers by application of 1 mM $\text{Ca}^{2+}$ in the rigor solution. In other experiments, the average value of $R_1/R_0$ from the separate experiments (0.096, see above) was used for the calculation, as indicated by parentheses in column 5. (Note that the exact value of $R_1/R_0$ is not very critical for the estimate of resting $[\text{Ca}^{2+}]$.) The average value of $[\text{Ca}]_D$, thus estimated, was 0.040 in 2 mM EGTA solutions and 0.052 in 10 mM EGTA.
solutions. Although the two sets of fCa values obtained with 2 mM EGTA and 10 mM EGTA were not statistically different, the slightly higher average value with 10 mM EGTA could be a result of the more complete buffering of intracellular Ca2+ by the high concentration of EGTA (see above). On the basis of the fCa values estimated in the presence of 10 mM EGTA and the dissociation constant of 1.0 μM (above), the resting [Ca2+], was calculated to be 54.7 ± 13.5 nM (see Discussion for the final estimate).

**DISCUSSION**

**Novel Calibration Method of the Intracellular Indicator with Cell Membrane Permeabilization**

A number of studies on many Ca2+ indicators have shown that the indicator properties are likely to be altered in the cytoplasm, probably by binding to cellular macromolecules: fura-2 (Konishi et al., 1988; Uto, Arai, and Ogawa, 1991), indo-1 (Hove-Madsen and Bers, 1992; Westerblad and Allen, 1993; Baker et al., 1994), fluo-3 (Harkins et al., 1993), fura red (Kurebayashi et al., 1993), and aequorin (Blatter and Blinks, 1991). The present results suggest that fura-2 conjugated to dextran can still bind to cellular proteins. The dissociation constant of fura-2 is increased by a factor of ~2.5 by conjugation with dextran (0.52 μM for fura dextran vs. ~0.2 μM for fura-2). The fura dextran K0 appears to be further increased by a factor of ~2 in the cytoplasm, as the K0 of 1.0 μM was estimated in the β-escin-treated fibers (see above).

The advantage of the calibration described in this article is that all of the calibration parameters can be estimated in the fibers in a relatively straightforward manner by simply measuring the fluorescence ratio. Although we did not estimate the leak of cytoplasmic proteins from the β-escin-treated fibers in the present study, the slow decline of fura dextran F(360), with a rate very similar to that reported in the previous study (see Fig. 1 in Konishi and Watanabe, 1995), led us to assume that most of the cytoplasmic proteins were well preserved. Thus, the β-escin-permeabilized fiber can be used to control [Ca2+]i in the presence of cellular proteins. The frequently used in vivo calibration with Ca2+ ionophores suffers difficulties in frog skeletal muscle because of the permeabilization of the SR (see above). Moreover, the relatively slow permeation of Ca2+ across the cell membrane treated with the Ca2+ ionophore makes one wonder if the [Ca2+]i, is truly in equilibrium with the extracellular [Ca2+]. This is particularly a problem in estimating the dissociation constant. The disadvantage of the present method, on the other hand, is that cellular molecules of small to moderate size leak out of the fiber as a result of the irreversible permeabilization of the cell membrane; we cannot exclude the possible influences of some cytoplasmic substance(s) missing in the β-escin-permeabilized fibers. It is also possible that the fiber swelling after β-escin treatment (see above) causes the dilution of the cytoplasmic proteins, which might reduce the protein-indicator interactions. If this is the case, the values of R0 and K0 of the intracellular indicator, and therefore the resting [Ca2+], might be somewhat underestimated.
Estimation of $R_0$ and $R_1/R_0$ in the Permeabilized Fibers

The $R_0$ value measured in the permeabilized fiber was not significantly influenced by the small change in the bathing solution pH (6.8–7.2) and $[\text{Mg}^{2+}]$ (1–2 mM), by 10 mM carnosine, and by 10 mM creatine phosphate. However, a low MgATP concentration in the bathing solution (below 1–2 mM) caused a large increase in the $R_0$ (Fig. 4). Because this effect of MgATP was not observed in the in vitro solution (see Results), the change in $R_0$ probably results from the indirect effect of MgATP (or free ATP) on either the indicator fluorescence or the $[\text{Ca}^{2+}]_i$. We think that the change in $[\text{Ca}^{2+}]_i$ in low [MgATP] is unlikely because (a) the rate of $\text{Ca}^{2+}$ uptake by the SR should be low in the low ATP (which should decrease $R_0$, not increase it, by elevating the $[\text{Ca}^{2+}]_i$); (b) the bathing solutions (and probably also the intracellular solution) are heavily buffered by 2–10 mM EGTA, and the change of the $R_0$ in low [MgATP] is very similarly observed in solutions containing either 2 mM or 10 mM EGTA; and (c) the wavelength dependence of the fluorescence change with low [MgATP] is clearly different from the $\text{Ca}^{2+}$ difference spectrum (Fig. 5 D). Alternatively, the change in the $R_0$ might reflect the alteration in the interaction between fura dextran and cellular proteins. Because at least a part of intracellular fura dextran molecules appear to be bound to cellular proteins, conformational changes of the proteins induced by the dissociation of ATP from cellular binding sites might subsequently change the microenvironment of the indicator molecules bound to the protein. Additional studies are required to understand the source of the $R_0$ change in low [MgATP].

The second calibration parameter $R_1/R_0$ was estimated in the permeabilized fibers in the rigor condition. Although the depletion of ATP causes some unknown bias on the fluorescence signal, the wavelength dependence of the fluorescence change on addition of $\text{Ca}^{2+}$ was similar to the $\text{Ca}^{2+}$ difference spectrum measured in vitro (Fig. 5 D). In addition, intact fiber experiments showed that the $R_1$ could be as low as 0.15 when the $[\text{Ca}^{2+}]_i$ was increased by high frequency stimulation in combination with the inhibited SR $\text{Ca}^{2+}$ pump (see Results). With the average $R_0$ value of 1.105 (Table II), the $R_1/R_0$ is calculated to be 0.136 or lower, a value consistent with that obtained in the permeabilized fibers (0.096).

Estimation of $K_0$ in the Permeabilized Fibers

Since the indicator $R$ quickly changes in response to a change in the bathing solution $[\text{Ca}^{2+}]$, the analysis shown in Fig. 7 assumed that $[\text{Ca}^{2+}]_i$ reached the same level as the bathing solution $[\text{Ca}^{2+}]$ at the quasi-steady state of the $R$. However, this assumption will be somewhat inaccurate if the potential inside the myofibrillar space is not zero (bath potential). The non-zero potential could arise from a Donnan equilibrium because of fixed negative charges in the myofibrils (Aldoroty and April, 1984; Godt and Baumgarten, 1984; Bartels and Elliott, 1985). Godt and Baumgarten (1984) used conventional electrodes in mechanically skinned fibers of frog soaked in a relaxing solution and measured $-4.4 \text{ mV}$ potential in the fiber. From the computation of the cytoplasmic constituents, Maughan and Godt (1989) predicted the Donnan potential of $-2.7 \text{ mV}$ for resting frog fibers. If the average of these values ($-3.6 \text{ mV}$) were to apply to our fibers, simple calculation of the Don-
nan equilibrium predicts that the intramyofibrillar Ca\(^{2+}\) concentration would be 33% higher than the extramyofibrillar concentration. It follows that the spatially averaged cytoplasmic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_c\), may be roughly 30% higher than the bathing solution [Ca\(^{2+}\)]\(_b\)], because the intramyofibrillar space occupies a large fraction of the fiber volume (83% of the fiber volume compared with 7% occupied by the extramyofibrillar space other than SR and mitochondria (Mobley and Eisenberg 1975)]. Godt and Baumgarten (1984) also measured the potential in ATP-free solutions and reported −6.5 mV for the rigor fibers. With this Donnan potential, the intramyofibrillar Ca\(^{2+}\) concentration would be 68% higher and [Ca\(^{2+}\)]\(_i\) would be ~60% higher than that in the bathing solution. If we take these values into account for the analysis shown in Fig. 7, the \(K_0\) values would be 1.3 μM for the rigor solutions (solid symbols) and 1.5 μM for the ATP containing solutions (open symbols): the average of these values, 1.4 μM, is 40% higher than the 1.0 μM estimated above. However, the myofilament charges might not fully influence the Ca\(^{2+}\) indicator molecules between the myofilament separated by ~40 nm, which is a distance much greater than the Debye length (~1 nm). Furthermore, if the anionic indicator molecules are partly excluded from the negatively charged myofilament space, the fluorescence from the intramyofibrillar space would have a smaller contribution to the total fluorescence signal than that expected from its volume. Therefore, the [Ca\(^{2+}\)]\(_i\), calculated from \(f_{CaD}\) values in Table II may be elevated by at most, 40%.

**Comparison of the \(K_0\) Values Estimated with the Permeabilization Method and an Alternative Technique**

The \(K_0\) value estimated in the permeabilized fibers, 1.0–1.4 μM as described above, may be compared with the value estimated with another method previously used for other indicators in other laboratories, namely, from kinetic fits of the indicator signal to \(\Delta[Ca^{2+}]\) in intact fibers (Baylor, Hollingworth, Hui, and Quinta-Ferreira, 1985; Klein et al., 1988; Baylor and Hollingworth, 1988). Fig. 8 illustrates an example of such an analysis in which the time course and the amplitude of the fura dextran fluorescence signal (calibrated as \(f_{CaD}\)) are compared with \(\Delta[Ca^{2+}]\) measured with furaptra in a separate experiment. Under the assumption of homogeneous distribution of Ca\(^{2+}\) and the indicator, the method permits estimation of a set of rate constants (\(K_+\) and \(K_-\)) and hence \(K_0\) (=\(K_-/K_+\)). The fura dextran fluorescence signals measured in five fibers were fitted to each of four \(\Delta[Ca^{2+}]\)'s obtained from four different fibers. The average values of \(K_+\), \(K_-\), and \(K_0\) in total of 20 combinations were 3.2 (±0.3) \(\times 10^7\) M\(^{-1}\)s\(^{-1}\), 80 (±14) s\(^{-1}\), and 2.5 (±0.3) μM, respectively. Thus, the kinetic fits give approximately two times larger \(K_0\) values than that estimated above from the permeabilized fibers (1.0–1.4 μM). There are several possible sources of errors in the kinetic fits: (a) the principal assumption of the method, uniform intracellular distribution of Ca\(^{2+}\), is unlikely during action potential stimulation (Cannel and Allen, 1984); (b) intracellular distribution of the indicator molecules may not be uniform; (c) the method includes indirect comparison of the furaptra and fura dextran signals measured in different muscle fibers (note that the similarity of the excitation wavelength band for the two indicators pre-
includes a simultaneous measurement); and (d) the estimated value of $K_0$ depends critically on the size and the time course of $\Delta[Ca^{2+}]_i$ measured with furaptra (Konishi, Hollingworth, Harkins, and Baylor, 1991), and the calibration of furaptra signals further relies on the calibration of purpurate diacetic acid (Hirota, Chandler, Southwick, and Waggoner, 1989; Konishi and Baylor, 1991).

We now have two estimates of the $K_0$ of fura dextran in the fiber interior: 1.0-1.4 $\mu M$ obtained in the permeabilized fibers, and 2.5 $\mu M$ obtained from the kinetic fitting. Although we tend to put more reliance on the former value because of some uncertainties related to the kinetic fitting (as discussed above), we cannot exclude the possibility that the permeabilized fibers report erroneously low $K_0$ as a result of the loss of some unidentified cytoplasmic molecules. Thus, it would be appropriate to conclude that the $K_0$ of fura dextran in the fiber for intracellular $Ca^{2+}$ is within the range of 1.0-2.5 $\mu M$. Consequently, the resting $[Ca^{2+}]_i$ calculated from the average $f_{CaD}$ in Table II ranges from 55 to 137 nM, as our best estimate from fura dextran.

**Resting Cytoplasmic $[Ca^{2+}]_i$ in Frog Skeletal Muscle Fibers**

Kurebayashi, Harkins, and Baylor (1993) estimated the resting $[Ca^{2+}]_i$, in intact single fibers of the frog *Rana temporaria* with fura red. For the calibration, they used the combination of the indicator absorbance and fluorescence measured from the fiber and in vitro solutions containing aldolase, which is an abundant soluble pro-
tein of muscle fiber. The indicator $K_0$ was estimated either in the in vitro solutions or by the kinetic fitting as carried out in the present experiment (Fig. 8). Their estimate of $[\text{Ca}^{2+}]_i$ ranged from 0.18 to 0.27 $\mu$M, which lies above any of the values reported by other methods (see Introduction). Harkins, Kurebayashi, and Baylor (1993) used fluo-3 in the same preparation (single fibers of Rana temporaria). Their calibration relied on both absorbance measurements in the fiber and fluorescence changes during activity, and the $K_0$ was estimated in a way similar to that used for fura red. The $[\text{Ca}^{2+}]_i$ range of 0.10-0.24 $\mu$M was estimated. These authors concluded that resting $[\text{Ca}^{2+}]_i$ in frog fibers is at least 0.1 $\mu$M and possibly as large as 0.3 $\mu$M. On the other hand, Blatter and Blinks (1991) applied aequorin and $\text{Ca}^{2+}$-sensitive microelectrodes to compare the estimates of $[\text{Ca}^{2+}]_i$ obtained with the two techniques in the same muscle fibers of R. temporaria. They proposed the $[\text{Ca}^{2+}]_i$ value of 38 nM (with aequorin) and 59 nM (with $\text{Ca}^{2+}$ microelectrodes) as an upper limit of resting $[\text{Ca}^{2+}]_i$, because they made measurements in the partially depolarized fibers (incubated in the Ringer's solution containing 12.5 mM K+) to raise resting $[\text{Ca}^{2+}]_i$ above the detection limit of their measurements. The authors of the above three studies all seem to have carefully set their criteria for healthy fibers. However, the estimated values of resting $[\text{Ca}^{2+}]_i$, form the upper end (Harkins et al., 1993; Kurebayashi et al., 1993) and the lower end (Blatter and Blinks, 1991) of the reported values in the literature. In the present study, we estimated the resting $[\text{Ca}^{2+}]_i$, only from fibers that underwent a 50-Hz tetanic stimulation for 1 s, because the local damage caused by either dissection or injection usually results in an incomplete response to long tetanic stimulation. Our estimated value of resting $[\text{Ca}^{2+}]_i$, 55–137 nM, lies between the two extremes, and supports the 50–100 nM range generally reported (or assumed) for the resting $[\text{Ca}^{2+}]_i$, in skeletal muscle fibers (e.g., Coray et al., 1980; Lopez et al., 1983). At this point, we do not have obvious explanations for the discrepancy between our range of estimates of resting $[\text{Ca}^{2+}]_i$ (0.06–0.14 $\mu$M) and either the higher range [0.1–0.3 $\mu$M reported by Kurebayashi et al. (1993) and Harkins et al. (1993)] or the lower range [<0.04–0.06 $\mu$M reported by Blatter and Blinks (1991)].

Westerblad and Allen (1993) used a different method to calibrate indo-1 in mouse skeletal muscle fibers. They injected a very high concentration of Ca$^{2+}$/EGTA solutions into muscle fibers containing indo-1 in an attempt to overcome the cellular intrinsic buffers and obtained the estimate of 26 nM for the average resting $[\text{Ca}^{2+}]_i$. (They also stated, as unpublished observations, that the resting $[\text{Ca}^{2+}]_i$, in Xenopus muscle calibrated with their method was about 40 nM [Westerblad and Allen, 1994b].) They found that $R_{\text{max}} / R_{\text{min}}$ (a parameter similar to our $R_1 / R_0$) is several fold smaller in the mouse fibers than in salt solutions, but they also found that the indicator $K_0$ in the fiber was very similar to that in the cell-free solutions. The latter finding appears to contradict the results from other laboratories, which have reported a substantial increase in the $K_0$ of indo-1 by interaction with intracellular proteins (Hove-Madsen and Bers, 1992; Baker et al., 1994).

Unless there are true differences in the $[\text{Ca}^{2+}]_i$ of frog and toad muscle fibers used in the different laboratories, the different estimates of $[\text{Ca}^{2+}]_i$ reveal calibration difficulties not yet identified for the $\text{Ca}^{2+}$ indicators and the other techniques, as pointed out by Kurebayashi et al. (1993).
The cell membrane permeabilization used here in combination with fura dextran should be applicable to other dextran-conjugated indicators (for Ca$^{2+}$ and other ions) in other types of cells. The present results suggest that the fluorescence ratio $R$ in the resting muscle fibers is close to the $R$ of Ca$^{2+}$-free indicator, and thus that the indicator $K_D$ is much higher than the resting [Ca$^{2+}$]. For the measurements of resting [Ca$^{2+}$], with better resolution, it is therefore desirable to use an indicator with a higher affinity for Ca$^{2+}$ than fura dextran in future studies.

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