Intracellular Calibration of the Calcium Indicator Indo-1 in Isolated Fibers of *Xenopus* Muscle

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ABSTRACT Estimates of the free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with fluorescent dyes are complicated by the fact that some properties of these dyes are altered in the intracellular environment. In the present study indo-1 was used to measure $[Ca^{2+}]_i$ in isolated muscle fibers from *Xenopus* frogs. Fluorescent ratio signals obtained from indo-1 were converted into $[Ca^{2+}]_i$ by means of an intracellular calibration method, which involved microinjection of 0.5 M EGTA and 1 M $CaCl_2$ to get the ratio at very low (R_{min}) and high (R_{max}) $[Ca^{2+}]$, respectively; ratios at intermediate $[Ca^{2+}]$ were obtained by injection of solutions with different EGTA/Ca^{2+}-EGTA proportions. This calibration gave an intracellular Ca^{2+} dissociation constant of indo-1 of 311 nM and a $[Ca^{2+}]_i$ at rest of 52 ± 4 nM (mean ± SE; n = 15). Indo-1 records during twitches were compared with records obtained with the much faster indicator mag-indo-1. This analysis suggests a Ca^{2+} dissociation rate of indo -1 of 52 s^{-1} (22°C). This makes indo-1 less suitable for measurements of $[Ca^{2+}]_i$ during twitches, whereas it is fast enough to follow most aspects of $[Ca^{2+}]_i$ during tetani, including the relaxation phase.

INTRODUCTION

There are several methods available to measure the free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in muscle: for example, aequorin (Blinks et al., 1978), Ca^{2+}-selective microelectrodes (López et al., 1983), metallochromic dyes (e.g., arsenazo III and antipyrylazo III) (Baylor et al., 1982); and fluorescent indicators excited by UV light (e.g., fura-2 and indo-1 (Grynkiewicz et al., 1985) or by visible light (e.g. fluo-3) (Minta et al., 1989). Although most of these methods readily detect changes of $[Ca^{2+}]_i$ (if these are not too rapid), it is more difficult to assess the absolute value of $[Ca^{2+}]_i$ because the function of most indicators is altered in the intracellular environment, possibly because of protein binding (Konishi et al., 1988; Blatter and Blinks, 1991; Kurebayashi et al., 1993). This means that calibrations performed in simple salt solutions will not be valid; for instance, the Ca^{2+} dissociation constant (K_D) of most fluorescent indicators has been found to be markedly higher than in simple salt solutions in the intracellular environment (e.g., Konishi et al., 1988; Kurebayashi et al., 1993). These difficulties in calibrating Ca^{2+} signals probably contribute to the large discrepancies between recent estimates of resting $[Ca^{2+}]_i$ in frog muscle, which range from less than 50 nM (Blatter and Blinks, 1991) to ~300 nM (Kurebayashi et al., 1993; Baker et al., 1994).

The kinetics of various Ca^{2+} indicators are considered to be slowed by constituents in the intracellular environment. For example, estimates in frog muscle fibers reveal at least a fourfold reduction of the Ca^{2+} dissociation rate (K_{off}) of fura-2 (Baylor and Hollingworth, 1988; Klein et al., 1988).

In the present study we have used indo-1 to measure $[Ca^{2+}]_i$ in intact, single fibers from *Xenopus*. We used an intracellular calibration method, based on microinjection of solutions with different $[Ca^{2+}]$ (Westerblad and Allen, 1992, 1993, 1994) to establish the intracellular K_D. Kinetics of indo-1 were assessed by comparison of indo-1 transients that are due to action potential stimulation with those obtained with the much faster indicator mag-indo-1. In agreement with published results, we found a substantial reduction of K_{off}, whereas the increase of K_D was markedly less than many previous estimates with fluorescent dyes.

METHODS

Experiments were performed on intact, single fibers from lumbrical muscles of adult female *Xenopus laevis*. The frogs were killed by stunning followed by decapitation. A detailed description of dissection, mounting, and stimulation has been given elsewhere (Lännergren and Westerblad, 1986). Fibers were bathed at room temperature (22°C) in a standard Ringer solution (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; NaH_2PO_4, 2.4; Na_2HPO_4, 0.6; pH = 7.0. Fibers were stimulated either with single pulses or with 350-ms trains of stimuli at 70 Hz. Force and fluorescence signals were displayed on a chart recorder and also stored in a computer for later analysis. Values are presented as mean ± SE. Student's t-tests were used to verify statistical significance; the significance level was set at 0.05 throughout.

We used fibers of two different types: large, transparent type 1 fibers and somewhat smaller, granular type 2 fibers (Lännergren and Smith, 1966). In addition to the difference in microscopic appearance, there are many other differences between the properties of these two fiber types. For instance, type 1 fibers are generally faster than type 2 fibers (Lännergren et al., 1982), and the twitch contraction time (measured as the time between onset of force development and peak force) of the present type 1 fibers was significantly shorter than that for the type 2 fibers (22.0 ± 0.8 ms (n = 4) compared with 25.5 ± 0.5 ms (n = 8)).

Fluorescence measurements

After being mounted and given a few test contractions, fibers were pressure injected with 10 mM of the potassium salts of indo-1 or mag-indo-1

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(Molecular Probes, Eugene, OR) in 150 mM KCl following a procedure described previously (Westerblad and Allen, 1992). After injection fibers were allowed to rest for at least 30 min, and thereafter the contractile function was tested. A few fibers did not respond to electrical stimulation after the rest period and were rejected. However, the vast majority of fibers displayed contractions almost identical to those before injection.

The optical arrangements for detection of the fluorescent light were the same as those described by Lee et al. (1991), except that the illumination wavelength was kept constant at 360 ± 5 nm and the emitted light was guided to two photomultiplier tubes with interference filters at 400 ± 5 and 505 ± 5 nm in front of their photocathodes. The signals from the photomultiplier tubes were fed into an analog divide circuit whose output provided a continuous 400/505 ratio signal. Fibers were constantly illuminated with red light (wavelengths > 600 nm), and this light was directed to a video camera. In this way fibers could be viewed throughout experiments; this illumination had no effect on fluorescence measurements.

We minimized photobleaching by keeping periods of illumination as short as possible, and the illuminating light was reduced 30-fold by a neutral-density filter. With these precautions there was no trend toward changes in the fluorescence ratio within the experimental duration.

We established the intracellular concentration of indo-1 by comparing the fluorescence of injected fibers with that of glass capillaries with similar inner diameters and filled with known concentrations of indo-1. The mean intracellular indo-1 concentration obtained in this way was 19 μM (range 5–41 uM). This concentration did not significantly buffer $[Ca^{2+}]_i$ transients, as judged from very similar contractile function before and after injection of indo-1 (see also Baylor and Hollingworth, 1988).

Calibration of indo-1

The $[Ca^{2+}]_i$-dependent signal of indo-1 was obtained as the ratio of the fluorescence signal at 400 nm to that at 505 nm. Intracellular calibration experiments were performed to convert ratios to $[Ca^{2+}]_i$ following a procedure originally developed to calibrate the Mg^{2+} indicator furaptra (Westerblad and Allen, 1992) and thereafter modified for calibration of indo-1 in mouse muscle fibers (Westerblad and Allen, 1993). This method is based on pressure injection of solutions with different $[Ca^{2+}]$; the video image of fibers was used to follow injections, and electrodes that were blocked either were replaced or their extreme tips were broken against the bottom of the chamber. For these calibration experiments we generally used blunt microelectrodes (resistance ~5 MΩ, compared with ~30 MΩ for standard injections of indo-1) so that relatively large amounts could be injected during each pressure pulse. The ratio at very low $[Ca^{2+}]_i$ (R_{min}) was obtained by injection of 0.5 M EGTA; 1 M $CaCl_2$ was injected to get the ratio at high $[Ca^{2+}]_i$. Ratios at intermediate $[Ca^{2+}]$ were obtained by injecting solutions where the proportion of [EGTA] to [Ca^{2+}-EGTA] was varied with the total EGTA concentration kept constant at 200 mM. All these solutions also contained 500 mM HEPES, and pH was set to 7.0 to match the intracellular pH (pH_i) of the present fibers (Westerblad and Lännergren, 1988). This gives an apparent Ca^{2+} K_D of EGTA of 400 nM (Fabiato, 1988).

Ratios were converted to $[Ca^{2+}]_i$ following the procedure developed by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_D \beta [(R - R_{min})/(R_{max} - R)], \quad (1)$$

where β is the ratio of the 505-nm signals at very low and saturating $[Ca^{2+}]_i$.

Estimation of indo-1 kinetics

We performed an analysis of the intracellular kinetics of indo-1 by comparing twitch records obtained with indo-1 with those obtained with mag-indo-1, which is a low-affinity Ca^{2+} indicator that responds to changes in $[Ca^{2+}]_i$ without any significant kinetic delays (cf. Konishi et al., 1991). For this analysis we converted indo-1 ratios into fractional Ca^{2+} occupancy of the dye (F), using the following equation (cf. Fig. 3 of Kurebayashi et al., 1993):

$$F = \left[1 + \frac{(R_{max} - R)}{\beta (R - R_{min})}\right]^{-1}. \quad (2)$$

Indo-1 records were often low-pass filtered at a cutoff frequency (f_c) of 100 Hz by use of a standard resistor–capacitor filter. For all kinetic analyses and corrections we removed the effect of this filter by using the following equation:

$$F = F_0 + \frac{dF_0/dt}{2\pi f_c}, \quad (3)$$

where F_0 is the recorded fractional Ca^{2+} occupancy. In a control experiment twitches were also recorded with a 3-kHz low-pass filter. The time courses of records with 3-kHz filtering and deconvoluted 100-Hz filtering were virtually identical.

Mag-indo-1 records were obtained from twitch stimulations, and no low-pass filtering was used. Each mag-indo-1 record represents the mean of 10 twitches. To reduce artifacts caused by fiber movement, fibers were stretched so that the sarcomere length was increased from ~2.3 μm to maximally 3.4 μm. Ca^{2+} binding to troponin C is affected by the number of strongly attached cross-bridges (e.g. Güth and Potter, 1987); hence severe stretching of the fiber might alter the $[Ca^{2+}]_i$ transient during a twitch (see Konishi et al., 1991). We therefore kept stretching to a minimum, and twitch force was never reduced to more than half of the original. Mag-indo-1 records are presented as the 400/505-nm ratio. No attempts were made to convert ratios into $[Ca^{2+}]_i$, but during twitches changes of the fractional Ca^{2+} occupancy of the dye are very small and hence there is a linear relation between the ratio and $[Ca^{2+}]_i$. Thus, the time courses of ratio and $[Ca^{2+}]_i$ transients will be the same.

RESULTS

Intracellular calibration

Fig. 1 *A-E* shows original records from intracellular calibration experiments performed in five different fibers. In Fig. 1 *A* a fiber was injected with 0.5 M EGTA to get R_{min}. The initial injection resulted in a marked reduction of the ratio, and the following injections gave some further decrease, until a reasonably stable level was obtained. R_{min} established in nine fibers was 0.095 ± 0.008.

Fig. 1 *B* shows injection of a solution with $[Ca^{2+}]$ set to 70 nM. Injection of this solution had little effect on the ratio signal, except for the transient increases associated with very large injections toward the end of the experiment. These transients are probably artifacts associated with the pressure pulses, because similar transients were observed with blocked microelectrodes (e.g., end of first trace of Fig. 1 *D*). The exact origin of these artifacts is not known; one possibility is the large movements of the injection microelectrode associated with the high-pressure pulses of long duration used during calibration. The ratio obtained with injection of 70 nm Ca^{2+} was 0.173 ± 0.003 (n = 3), which compares to 0.137 ± 0.007 before injection in the same fibers. Thus, resting $[Ca^{2+}]_i$ seems, if anything, to be lower than 70 nM.

Fig. 1 *C* shows an experiment in which a solution with 200 nM Ca^{2+} was injected. The fiber developed a transient contracture with increased $[Ca^{2+}]_i$ when it was penetrated

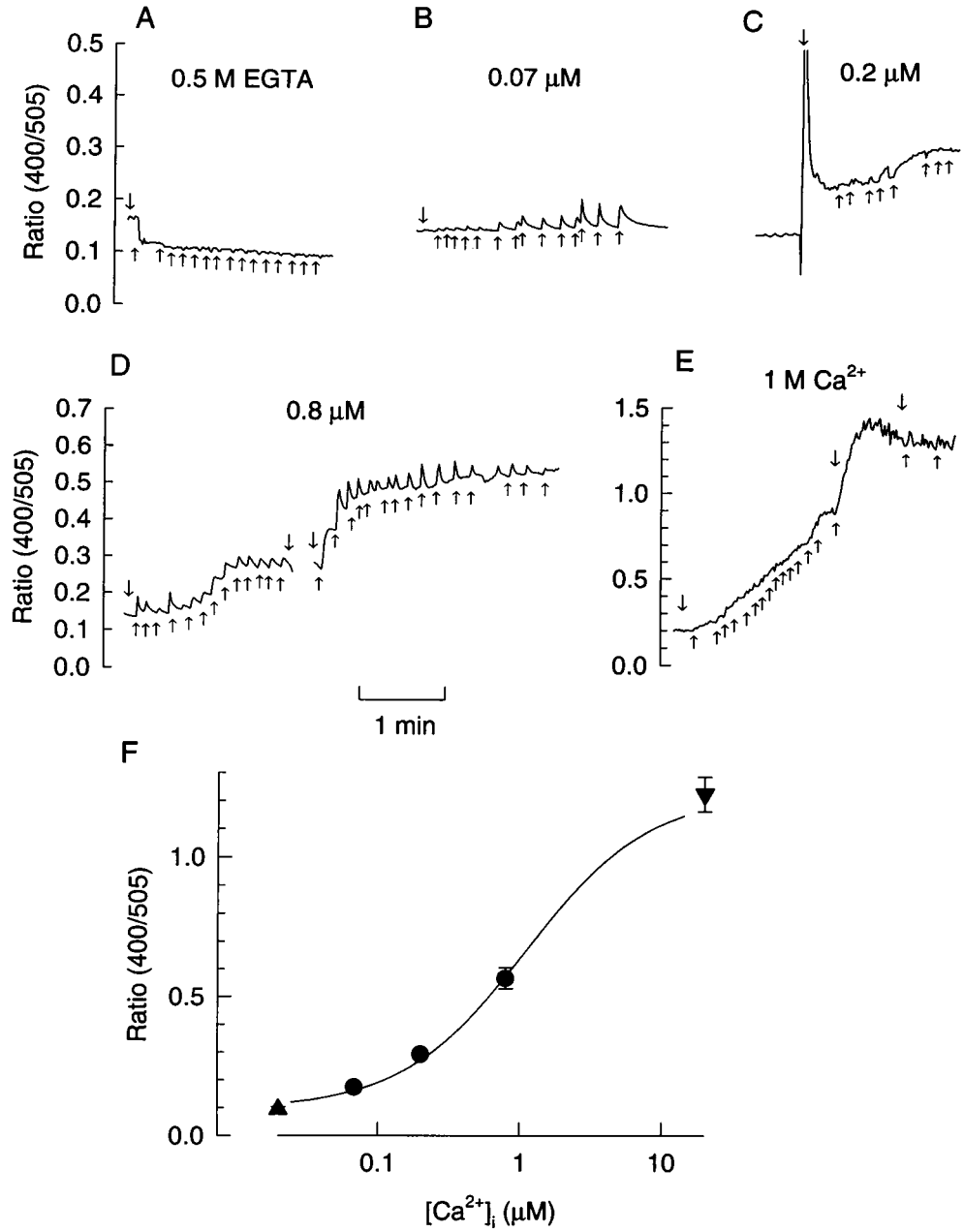

FIGURE 1 Original fluorescent ratio records from periods of pressure injection of (*A*) 0.5 M EGTA to get R_{min} and mixtures of EGTA/Ca^{2+}-EGTA that give [Ca^{2+}] of (*B*) 0.07 μM, (*C*) 0.2 μM, and (*D*) 0.8 μM and (*E*) of 1 M $CaCl_2$ to get R_{max}. Downward arrows indicate times of microelectrode penetration or withdrawal (second arrow in *D* and *E*). Upward arrows show times where pressure pulses were applied; the duration of pressure pulses was generally increased during experiments. The time bar in *D* refers to *A–E*. (*F*) Mean values (±SE; error bars mostly smaller than symbols) of R_{min} (▲), R_{max} (▼), and ratios at intermediate [Ca^{2+}] (●). The curve was drawn by a least-squares fit of Eq. 1 to the data at intermediate [Ca^{2+}] and, with the measured values of R_{min} (0.095), R_{max} (1.23), and β (3.50), this gives an intracellular K_D for indo-1 of 311 nM.

with a relatively blunt microelectrode. The ratio then fell to a level that was higher than that before penetration. Injection of the 200 nM Ca^{2+} solution increased the ratio further, until a stable level was obtained at a ratio of 0.3. Similar results were obtained in two more fibers into which 200 nM Ca^{2+} was injected starting from the resting ratio. Furthermore, one fiber accidentally developed a contracture during a tetanus elicited immediately before injection. In this fiber injections started at a ratio of ~0.6, and injection of the 200 nM solution brought the ratio down to 0.28, which was thus similar to the ratios obtained when one starts at a low ratio. The ratio at 200 nM [Ca^{2+}] in the four fibers was 0.293 ± 0.011. At this stage none of the fibers produced a noticeable contracture.

Three fibers were injected with 800 nM Ca^{2+}. This [Ca^{2+}] is well above the contraction threshold; hence a contracture will develop during injection that might damage the fiber. To reduce the risk of fiber damage, 20 mM

2,3-butanedione monoxime was added to the bath solution ~10 min before injections. This drug is known to inhibit Ca^{2+}-induced force production (e.g., Horiuti et al., 1988), and it abolished contractures associated with injections. One experiment with injection of 800 nM Ca^{2+} is shown in Fig. 1 *D*. Initially pressure pulses resulted in clear injections (as judged from viewing the fiber), and the fluorescence ratio increased. However, the electrode was blocked after ~10 injections, and no further increase of the ratio was observed (each pressure pulse still resulted in a transient increase of the ratio, but this was probably due to movement of the injection microelectrode associated with the pressure pulse). The electrode was then withdrawn from the fiber, and the outermost part of the tip was broken against the bottom of the experimental chamber. The fiber was again penetrated, subsequent pressure pulses resulted in clear injections, and the ratio increased until a stable level was reached. The stable ratio with 800-nM injection was 0.57 ± 0.04 (n = 3).

R_{max} was established by injection of 1 M $CaCl_2$. At this high [Ca^{2+}] massive contractures will develop. In a few early experiments we took no action to reduce the contracture, and injection of $CaCl_2$ then gave a rapid contracture that developed while the fiber was penetrated by the microelectrode. This resulted in fiber destruction and rapid loss of fluorescence signal, which made estimates of R_{max} unreliable. To minimize this problem we injected $CaCl_2$ into fibers that previously had been injected with EGTA, and in some experiments 20 mM 2,3-butanedione monoxime was added to the bath solution. There were two reasons for using fibers already injected with EGTA to establish R_{max}. First, injection of EGTA and $CaCl_2$ into the same fiber allowed estimation of the parameter β of Eq. 1 (see below). Second, in these fibers contractures that were due to $CaCl_2$ injection developed more slowly, which meant that there was time to withdraw the microelectrode before the fiber was destroyed. Addition of 2,3-butanedione monoxime caused a further reduction of the amplitude and slowing of the rising phase of the Ca^{2+}-induced contracture. When the contracture was stable, the fiber was again penetrated, and additional injections were performed. Fig. 1 *E* shows records from a fiber for which R_{max} was assessed. Injections initially gave a gradual increase of the ratio, but the ratio started to increase more rapidly at ~0.7, and this was associated with a marked contracture. The electrode was removed after the injection at a ratio of ~0.9. Thereafter fiber movement was substantial, and the highest ratio was associated with a large movement. The contracture then became stable, and the fiber was again penetrated and two additional injections performed. The reasonably stable ratio after the end of the period of substantial fiber movement was taken as R_{max}. A reliable measure of R_{max} was obtained in seven fibers and was 1.23 ± 0.06.

Two methods were used to assess the parameter β of Eq. 1. In three fibers β could be obtained from the 505-nm signal at R_{min} and R_{max} after compensation for the general decline in fluorescence and amounted to 3.52 ± 0.21. We also used the method described by Bakker et al. (1993) to estimate β. Adapted to our experimental conditions, this means that we first made a plot of the 505- versus 400-nm fluorescence amplitude at various $[Ca^{2+}]_i$. β was then obtained from the slope of this plot (m) and the following equation (obtained by reorganizing Eq. 3 of Bakker et al.):

$$\beta = \frac{1 - mR_{max}}{1 - mR_{min}}. \quad (4)$$

This analysis was performed on fluorescence records from tetanic contractions of eight fibers and gave a β of 3.48 ± 0.27. Thus the two methods gave very similar values, and we have set β to 3.50 in what follows.

The curve in Fig. 1*F* was drawn by a least-squares fit of Eq. 1 to the measured mean ratios at 70 200, and 800 nM Ca^{2+}. With the measured values of R_{min}, R_{max}, and β, this gives an intracellular K_D for indo-1 of 311 nM.

Measurements of $[Ca^{2+}]_i$ at rest and during tetani

The resting fluorescence ratios were obtained from periods at least 3 min after a contraction, and no obvious change of the resting ratio was observed during experiments. The resting ratio was 0.147 ± 0.004 (n = 15), which, with the above calibration values, corresponds to a fractional Ca^{2+} occupancy of indo-1 of 0.144 ± 0.010 and a $[Ca^{2+}]_i$ of 52 ± 4 nM. No significant difference in the resting ratio between type 1 and type 2 fibers was observed.

The tetanic ratio, measured as the mean ratio during the final 100 ms of stimulation, was 0.88 ± 0.02 (n = 15), and the corresponding tetanic $[Ca^{2+}]_i$ was 2.6 ± 0.3 μM. The tetanic ratio was not significantly different in type 1 and type 2 fibers. The mean tetanic ratio is considerably lower than R_{max} and translates to a fractional Ca^{2+} occupancy of indo-1 of 89%. To study whether ratios closer to R_{max} could be obtained during tetani, we tried three interventions that would increase tetanic $[Ca^{2+}]_i$: prolonged tetanic stimulation because ratios generally show a gradual increase during tetani (see Fig. 4 *D* and *E*); 10 standard tetani given at 4-s intervals because we previously observed a marked increase of tetanic $[Ca^{2+}]_i$ at the onset of fatigue produced by repeated tetani (Allen et al., 1989); and application of 0.5 mM caffeine because low doses of caffeine have been shown to increase action-potential-induced Ca^{2+} release from the sarcoplasmic reticulum (Konishi and Kurihara, 1987). These three interventions gave similar results, and ratios of ~1.1 were regularly observed; in some experiments ratios were as high as 1.15, which corresponds to a fractional Ca^{2+} occupancy of indo-1 of 98%.

Estimation of indo-1 kinetics

It is possible to estimate the intracellular kinetics of a Ca^{2+} indicator by comparing its signal with "real" $[Ca^{2+}]_i$ tran-

sients obtained with a very fast indicator (Baylor and Hollingworth, 1988; Klein et al., 1988; Kurebayashi et al., 1993). In this study we compared twitch records obtained with indo-1 and the very fast, low-affinity indicator mag-indo-1 (Fig. 2). Fig. 2 *A* shows records of the fractional Ca^{2+} occupancy of indo-1 (*F*) from twitches produced in two fibers, one type 1 and one type 2 fiber. In twitches indo-1 reached ~80% saturation with Ca^{2+}, and there was no marked difference between fiber types in this respect. The time course of the transient was, however, markedly faster in the fast type 1 fibers than in the slower type 2 fibers: time to peak 7.8 ± 0.9 versus 10.3 ± 1.3 ms and half-width 37.0 ± 4.5 versus 50.8 ± 2.1 ms. This indicates that the kinetics of indo-1 are clearly fast enough that differences in $[Ca^{2+}]_i$ transients can be detected during twitches.

Mag-indo-1 transients were recorded in four type 2 fibers, and one representative example is shown in Fig. 2 *B*. The time course of the mag-indo-1 transient is markedly faster than those obtained with indo-1. The twitch force is also shown in Fig. 2 *B*, and it can be seen that the mag-indo-1 transient reached its peak before force started to develop (cf. Claflin et al., 1994). The time to peak and the half-width of mag-indo-1 transients were 3.8 ± 0.3 and 7.4 ± 0.6 ms, respectively (n = 4). These values are similar to other estimates in frog muscle; for example, Kurebayashi et al. (1993) give a time to peak of 6 ms and a half-width of 10 ms in twitches produced in frog fibers at a slightly lower temperature (16 rather than 22°C).

Indo-1 and mag-indo-1 transients were recorded in two groups of fibers. If the real $[Ca^{2+}]_i$ transients were different in the two groups, this would introduce an error in the analysis of indo-1 kinetics. To minimize this risk we used only fibers of one specific type (type 2 fibers) in the analysis, and twitch force characteristics were very similar in the two groups: time to peak force 26 ± 0.8 versus 25.0 ± 0.5 ms and half-relaxation time 24.8 ± 1.3 versus 26.0 ± 1.4 ms. We used a standard curve-fitting routine (Sigmaplot, Jandel Scientific, Corte Madera, CA) and fitted fractional Ca^{2+} occupancy records of indo-1 to an average $[Ca^{2+}]_i$ transient obtained with mag-indo-1, using the following equation (obtained by reorganizing Eq. 4 of Klein et al., 1988):

$$[Ca^{2+}]_i = \frac{dF/dt + K_{off}F}{K_{on}(1 - F)}. \quad (5)$$

In this analysis the peak of the $[Ca^{2+}]_i$ transient was arbitrarily set to 100%; it should be noted that simply changing the amplitude of the transient affects only the Ca^{2+} association rate (K_{on}), which is inversely proportional to the amplitude.

Fig. 3 shows the outcome of the analysis in one fiber. The agreement between the mag-indo-1 and the fitted transient was similar in the other fibers, and the analysis gave a Ca^{2+} dissociation rate of indo-1 (K_{off}) of 52 ± 4 s^{-1} (range 40–60 s^{-1}).

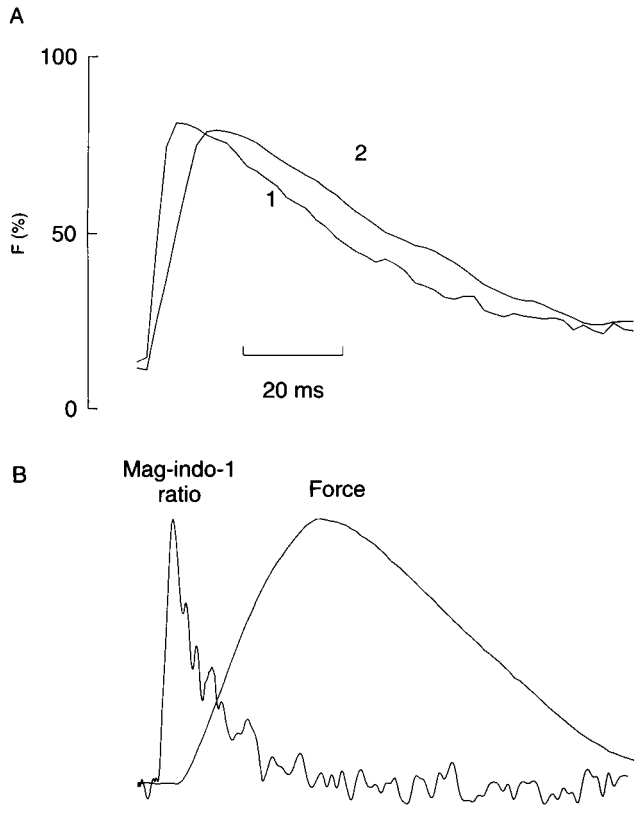

FIGURE 2 (*A*) Records of the fractional Ca^{2+} occupancy of indo-1 obtained in response to a single stimulus pulse in a type 1 fiber (*1*) and a type 2 fiber (*2*). Observe the faster time course in the type 1 fiber. (*B*) Mag-indo-1 fluorescence ratio (400/505 nm) obtained in a type 2 fiber in response to twitch stimulation and the accompanying force. Records are scaled to yield a similar amplitude. The time scale is the same as in *A*.

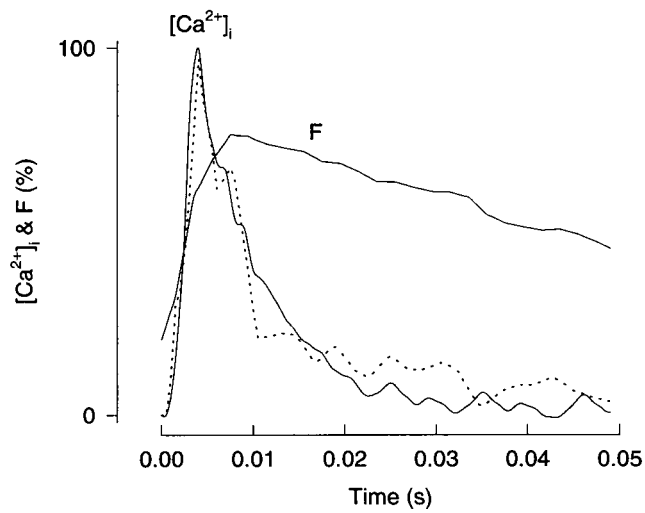

FIGURE 3 Estimation of the intracellular K_{off} of indo-1. The fractional occupancy record (*F*) of a single type 2 fiber was curve fitted by Eq. 5 to a $[Ca^{2+}]_i$ transient obtained from the mean mag-indo-1 signal of four type 2 fibers. The peak of the $[Ca^{2+}]_i$ transient is arbitrarily set to 100%. The dotted curve shows the result of the curve fit; K_{off} in this fiber was 46 s^{-1}. The stimulus pulse was given at time 0.

An analysis of indo-1 kinetics was also performed in which an F function was driven by the mag-indo-1-derived $[Ca^{2+}]_i$ transient and fitted to the observed F signal (cf. Baylor and Hollingworth, 1988; Klein et al.,1988). This analysis gave a mean K_{off} of 54 s^{-1}; thus the two curve-fitting methods gave very similar results.

Kinetic correction of indo-1 signals

Eq. 5 was used to produce $[Ca^{2+}]_i$ transients that were corrected for the kinetics of indo-1, and Fig. 4 shows examples of this. Analyses were performed mainly with K_{off} set to 52 s^{-1}, i.e., the mean value obtained with curve fitting; twitch records were also produced with K_{off} set to 40 and 60 s^{-1} (i.e., the range). The K_{on} was in all cases set so that the K_D was kept constant at 311 nM, which was the value obtained in the steady-state calibration (see above). Records from a twitch produced in a type 1 fiber are shown in Fig. 4 *A-C*. It can be seen that, in addition to an increased noise level, the kinetic correction resulted in a faster $[Ca^{2+}]_i$ transient with an increased amplitude.

In type 1 fibers (n = 4) the time to peak $[Ca^{2+}]_i$ was 7.5 ± 0.5 ms without correction and 3.3 ± 0.3 ms with K_{off} set to 52 s^{-1}; corresponding values for the half-width were 18.3 ± 2.3 and 4.8 ± 0.3 ms. The time course was not significantly different with 40-, 52-, and 60-s^{-1} correction. The peak $[Ca^{2+}]_i$ was markedly lower without correction (1.3 ± 0.2 μM), and there was also a clear dependence on the K_{off} used: 5.42 ± 0.13, 4.31 ± 0.10, and 3.80 ± 0.09 μM with K_{off} set to 40, 52, and 60 s^{-1}, respectively. Kinetic correction gave the same general pattern in type 2 fibers, but compared with those in type 1 fibers the time courses were generally slower and the kinetically corrected values for peak $[Ca^{2+}]_i$ slightly lower.

Fig. 4 *D* and *E* shows records from a standard 70-Hz tetanus produced in a type 2 fiber. Kinetic correction of tetani revealed a brief $[Ca^{2+}]_i$ transient at the onset of contraction, which corresponds to the increased $[Ca^{2+}]_i$ amplitude in twitches. After the initial transient, uncorrected and corrected records were qualitatively similar, except for the increased noise introduced by correction. After the end of stimulation the uncorrected $[Ca^{2+}]_i$ lagged behind the corrected one by 1–2 ms (Fig. 4 *F*). We also measured the initial rate of $[Ca^{2+}]_i$ decline by fitting the first 40 ms to a single exponential; we chose this time to avoid the transient increase of $[Ca^{2+}]_i$ during relaxation described as the "bump" (Cannell, 1986). This rate was not significantly different in uncorrected and 52-s^{-1} records: 34.9 ± 2.6 and 36.7 ± 2.4 s^{-1}, respectively (n = 8).

DISCUSSION

Intracellular calibration

A major advantage of the present calibration method is that it is practically and theoretically simple, provided that the cells under study are accessible to microinjection. In fact, nonmuscle cells would be easier to use, inasmuch as the major problem with the present skeletal muscle preparation is that fibers developed a contracture at high $[Ca^{2+}]_i$. The complete calibration is performed in the intracellular environment and hence does not depend on the composition of in vitro solutions, which is the case for many other calibration methods (e.g. Konishi et al., 1988; Kurebayashi et al., 1993).

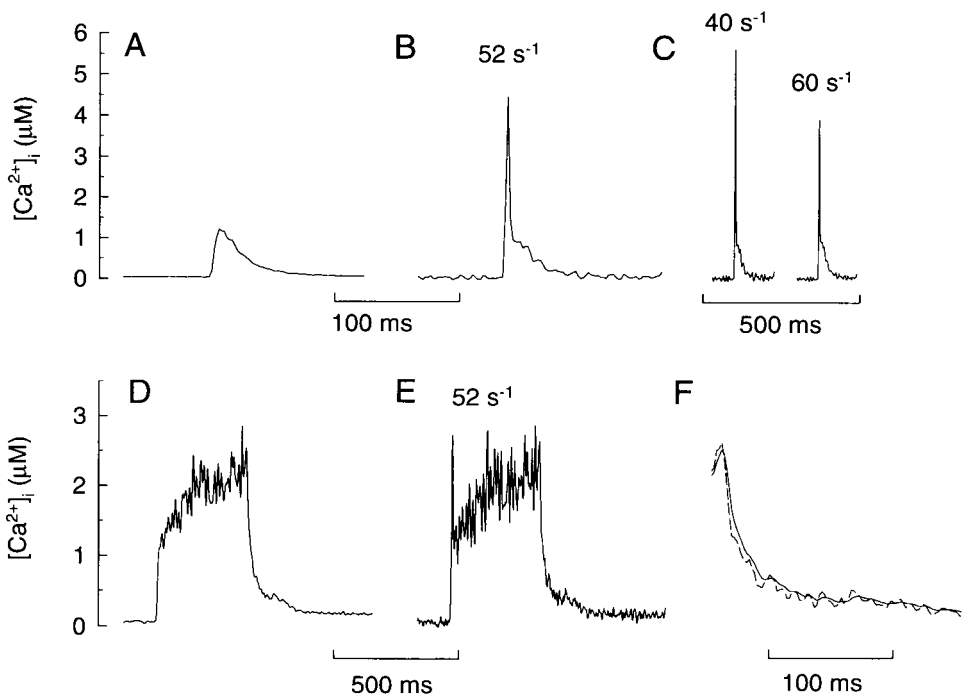

FIGURE 4 $[Ca^{2+}]_i$ records obtained from a twitch in a type 1 fiber (*A–C*) and from a tetanus in a type 2 fiber (*D–F*). *A* and *D* show $[Ca^{2+}]_i$ records without kinetic correction. For *B* and *E* records were corrected by use of Eq. 5 with K_{off} = 52 s^{-1} and K_{on} = 167 μM^{-1} s^{-1} (K_D = 0.311 μM, i.e. the value from steady-state calibrations). *C* shows $[Ca^{2+}]_i$ transients on a compressed time scale with K_{off} set to 40 and 60 s^{-1} and K_D kept constant at 0.311 μM. Observe the marked variation in peak amplitude with different K_{off}. *F* shows $[Ca^{2+}]_i$ during tetanic relaxation on an expanded time scale and compares the uncorrected record (continuous curve; from *D*) and the record with K_{off} set to 52 s^{-1} (dashed curve; from *E*). Records start at the last stimulus pulse of the tetani. Note that kinetic correction has no marked effect on $[Ca^{2+}]_i$ during tetanic relaxation.

Another advantage is that injections can be given until no further change in the ratio is obtained. Thus one can be reasonably confident that the final intracellular $[Ca^{2+}]$ equals that in the microelectrode. An alternative method to get an intracellular calibration is to make the membrane permeable to Ca^{2+} by using ionophores. With this method great care has to be taken to ensure that the intracellular Ca^{2+} is equilibrated with the extracellular Ca^{2+} (e.g., Roe et al., 1990), and the same extracellular $[Ca^{2+}]$ can give stable fluorescent ratios at different levels, depending on the experimental protocol (e.g., Fig. 2 of Ikenouchi et al., 1991).

One disadvantage of the present method is that a complete calibration experiment cannot be performed in a single cell. Thus the calibration curve must be constructed from mean values of many cells. This means that the method is not suitable if the parameters in Eq. 1 show large intercellular variation. In the present study this seemed not to be a serious problem because R_{min}, R_{max}, β, and the ratios at intermediate $[Ca^{2+}]$ showed little variation between experiments.

Another disadvantage is that the intracellular milieu will be altered by the injections: the injected solutions have high ionic strength and high osmolarity. The increased tonicity will result in water moving into the cell and fiber swelling. Because the calibration solutions do not contain any proteins, fiber swelling will result in a dilution of the intracellular proteins. The function of indo-1 is known to be affected by changes in ionic strength (Grynkiewicz et al., 1985; Williams and Fay, 1990; Uto et al., 1991) and protein binding (Konishi et al., 1988; Uto et al., 1991; Baker et al., 1994). Neither of these potential problems is easy to quantify, but we suspect that such effects are small for the following reason: Although injections were continued for some time, often a large fraction of the change in ratio occurred on the first injection when the changes in volume and ionic strength would have been minimal (see Fig. 1 *A*).

Establishing R_{max} was found to be problematic because fibers then developed a contracture and the overall fluorescence fell relatively fast. The highest ratio observed in tetanic contractions was 1.15. With an R_{max} of 1.23 this represents a maximal fractional Ca^{2+} occupancy of indo-1 of 98%, which seems reasonable.

We used two methods to assess β, and these gave similar results. Both methods depend on the accuracy of our estimates of R_{min} and R_{max}; for example, β is too high if R_{max} is overestimated or R_{min} underestimated. However, an error in β will affect our values of $[Ca^{2+}]_i$ neither at rest nor during tetani when $[Ca^{2+}]_i$ is changing relatively slowly, because the position on the $[Ca^{2+}]_i$ axis is decided by the points at intermediate $[Ca^{2+}]_i$ (Fig. 1 *F*); an increase of β will only result in a corresponding reduction of K_D and vice versa, because the $[Ca^{2+}]_i$ that corresponds to $(R_{min} + R_{max})/2$ is $K_D \times \beta$ (see Uto et al., 1991). However, estimates of the amplitude of rapid $[Ca^{2+}]_i$ transients are based on the value of K_D and will be affected by an error in β (see Fig. 4 and below).

We obtained an intracellular K_D of 311 nM, which should be compared with ~240 nM in simple salt solutions at pH 7.0 (Grynkiewicz et al., 1985; Westerblad and Allen, 1993). This intracellular increase of K_D is markedly smaller than many previous estimates made with both indo-1 ((Hove-Madsen and Bers, 1992; Baker et al., 1994)) and related dyes (e.g. Konishi et al., 1988; Kurebayashi et al., 1993). The cause of this difference is at present unclear. It is well established that binding to intracellular proteins reduces the Ca^{2+} affinity of most fluorescent Ca^{2+} indicators (Konishi et al., 1988; Hove-Madsen and Bers, 1992; Harkins et al., 1993; Kurebayashi et al., 1993; Baker et al., 1994), and this will certainly take place in our experiments. In three other recent studies attempts were made to establish the intracellular K_D of indo-1 in muscle cells. In the studies of Hove-Madsen and Bers (1992) and Baker et al. (1994) K_D was established in in vitro solutions with intracellular proteins, and large increases of K_D were observed compared with the values obtained in standard salt solutions. On the other hand, in the present study and in the study by Ikenouchi et al. (1991) K_D was assessed in the cells with injection and ionophores, respectively, and a K_D closer to that obtained in simple salt solutions was obtained. Thus there might exist some other constituent in the intracellular environment that counteracts the reduction of the sensitivity that is due to protein binding. For example, it is well established that the Ca^{2+} binding of fluorescent indicators is sensitive to ionic strength, so K_D increases with increasing ionic strength (e.g., Grynkiewicz et al., 1985; Williams and Fay, 1990; Uto et al., 1991). It is therefore possible that addition of proteins to salt solutions increases the ionic strength to a level that is higher than that in the myoplasm, but this issue is difficult to quantify because the contribution to the ionic strength of the immobile, multicharged myofilaments is uncertain (for a discussion see Godt and Maughan, 1988).

Intracellular kinetics of indo-1

We estimated the intracellular kinetics of indo-1 by fitting records of the fractional occupancy during twitches to a $[Ca^{2+}]_i$ transient obtained with the low-affinity Ca^{2+} indicator mag-indo-1. This analysis gave a K_{off} of ~52 s^{-1}, which compares with an in vitro value of ~160 s^{-1} at 22°C (Jackson et al., 1987; Lattanzio and Bartschat, 1991). Thus the intracellular K_{off} was reduced threefold, in reasonable agreement with the reduction observed with fura-2 (Baylor and Hollingworth, 1988; Klein et al., 1988).

An estimate of the intracellular K_D of indo-1 can be obtained from the kinetic analysis because K_D is the ratio of K_{off} to K_{on}. The value of K_{on}, and therefore also of K_D, depends on the amplitude of the $[Ca^{2+}]_i$ transient, which is uncertain; we did not perform any calibration of the mag-indo-1-derived $[Ca^{2+}]$ signal because this calibration has to be performed in vitro and is likely to be altered in the intracellular environment (e.g., Konishi et al., 1991). Kinetic correction of the indo-1 twitch records

with K_{off} set to 52 s-1 and K_D to 311 nM gave a peak $[Ca^{2+}]_i$ in twitches of 4.3 μM. This value is lower than many estimates in frog muscle, for which values of the peak $[Ca^{2+}]_i$ range from ~5 to ~10 μM (e.g., Konishi et al., 1991). If it is assumed that the true peak $[Ca^{2+}]_i$ in our fibers is close to the higher of these values (10 μM), then the K_{on} of indo-1 would be reduced more than twofold, resulting in an increase of K_D to ~700 nM; all values of $[Ca^{2+}]_i$ would be increased accordingly. Very similar results were recently presented by Konishi and Watanabe (1995), who used fura-2 dextran in frog muscle fibers and got a K_D 2.5 times higher with estimates of dye kinetics in twitches than in a steady-state calibration method in which the cell membrane was made permeable to small molecules by addition of β-escin. Those authors discussed possible sources of error in the kinetically established K_D, and these include nonuniform distribution of Ca^{2+} during twitches; comparison of records from two groups of fibers; and problems with calibration of the fast, low-affinity $[Ca^{2+}]_i$ indicator (for references see Konishi and Watanabe, 1995). When our peak $[Ca^{2+}]_i$ is related to the values given by Konishi et al. (1991) additional sources of error are introduced because we used another frog species (*Xenopus* rather than *Rana*) and worked at a higher temperature (22 instead of 16°C). Thus there are many problems involved in establishing K_D from estimates of dye kinetics in twitches and, although there are also problems with the present steady-state calibration, we tend to believe that the magnitude of the latter problems is smaller.

Estimates of $[Ca^{2+}]_i$ at rest and during tetani

The mean $[Ca^{2+}]_i$ at rest in our fibers was 52 nM. This is in reasonable agreement with many previous estimates in frog muscles (Weingart and Hess, 1984; Baylor and Hollingworth, 1988; Klein et al., 1988; Blatter and Blinks, 1991). However, recent studies by Baylor and co-workers (Kurebayashi et al., 1993; Harkins et al., 1993) using long-wavelength fluorescent indicators give markedly higher values of $[Ca^{2+}]_i$ at rest: at least 100 nM and maybe as high as 300 nM. Furthermore, Baker et al. (1994) suggest an even higher $[Ca^{2+}]_i$ at rest in frog muscle: ~350 nM.

We consider a resting $[Ca^{2+}]_i$ as high as ~300 nM unlikely in the present preparation on the following grounds. First, we previously depolarized fibers in solutions with increased K^+ concentrations, and this resulted in a clear increase of resting $[Ca^{2+}]_i$ without any increased force production (Lee et al., 1991). Based on skinned-fiber data from *Xenopus* (Stienen et al., 1987, 1993), an increase of $[Ca^{2+}]_i$ from a starting value of ~300 nM would result in clear force increases. Second, Ca^{2+} loading of skinned *Xenopus* fibers with functionally intact sarcoplasmic reticulum is close to maximal at a bath $[Ca^{2+}]$ of 100 nM (Stienen et al., 1993), a result that suggests a resting $[Ca^{2+}]_i$ below rather than above 100 nM. Third, injection of the EGTA/Ca^{2+}-EGTA solution with $[Ca^{2+}]$ set to 70 nM resulted in only a minor change of the fluorescence ratio, and, if anything, injections gave an increased ratio (Fig. 1 *B*), which indicates a resting $[Ca^{2+}]_i$ slightly lower than 70 nM. One possible source of error in these experiments is that EGTA/Ca^{2+}-EGTA solution does not set the $[Ca^{2+}]$ to 70 nM within the cell. For instance, Ca^{2+} buffering of EGTA is affected by the ionic strength (Fabiato, 1979). We have assumed an ionic strength of 0.16 M, which is close to an estimated value for frog muscle (0.14 M; Gordon et al., 1973). However, estimates of the intracellular ionic strength are uncertain (see Godt and Maughan, 1988), and a higher value will increase the apparent K_D of EGTA. If, for example, the intracellular ionic strength is twice the value that we assumed, the apparent K_D of EGTA will be increased from 400 to ~480 nM. This will give a proportional increase of the K_D for indo-1, and the mean resting $[Ca^{2+}]_i$ will increase from 52 to 62 nM. Thus, an error in the assumed ionic strength will have a relatively small effect.

Indo-1 did not become saturated during standard tetani, and increases of the ratio could easily be detected. Tetanic $[Ca^{2+}]_i$ was not significantly different in type 1 and type 2 fibers, and during the last 100 ms of 350-ms tetani we obtained a mean $[Ca^{2+}]_i$ of 2.6 μM. Of the parameters established in the calibration, the tetanic $[Ca^{2+}]_i$ is most sensitive to R_{max}, and a slight reduction of R_{max} would lead to a marked increase of tetanic $[Ca^{2+}]_i$; for example, a reduction of R_{max} to 1.15 (the highest ratio observed in tetanic contractions) will give a mean tetanic $[Ca^{2+}]_i$ under control conditions of 3.2 μM.

The tetanic $[Ca^{2+}]_i$ that we report (2.6 μM) is somewhat lower than many previous estimates in frog muscle (e.g. Cannell, 1986; Caputo et al., 1994). However, we used relatively brief tetani (duration 350 ms), and the tetanic $[Ca^{2+}]_i$ displayed a gradual rise during these contractions (see Fig. 4). With increased tetanus duration we regularly observed a tetanic $[Ca^{2+}]_i$ that was 4 μm higher, which lies within the range of previous estimates.

Usefulness of indo-1 to measure $[Ca^{2+}]_i$ during activation

The kinetics of indo-1 seem too slow to allow accurate measurements of peak $[Ca^{2+}]_i$ during twitches. As illustrated in Fig. 4, the peak $[Ca^{2+}]_i$ in a twitch critically depends on the kinetic correction, which is relatively uncertain. With kinetic correction it should, however, be possible to get the approximate time to peak $[Ca^{2+}]_i$ and half-width because these measures are less dependent on the value of K_{off} used for kinetic correction.

During tetanic stimulation the exact level of $[Ca^{2+}]_i$ is somewhat uncertain, for example because of problems involved in establishing R_{max} (discussed above). Furthermore, the high degree of Ca^{2+} saturation of indo-1 during tetani

will increase the noise in $[Ca^{2+}]_i$ records. The kinetics of indo-1, on the other hand, seem fast enough to follow $[Ca^{2+}]_i$ during tetani, except for the rapid transient of $[Ca^{2+}]_i$ at the onset of a tetanus (Fig. 4 *D* and *E*). During the plateau of a tetanus indo-1 acts as a low-pass filter and reduces the size of rapid transients. In experiments aimed at correlating $[Ca^{2+}]_i$ to force, this would not be a problem because the contractile apparatus will not respond to these rapid transients. During relaxation indo-1 introduces a short delay (1–2 ms), whereas the rate of $[Ca^{2+}]_i$ decline is not affected with a K_{off} of indo-1 of 52 s^{-1}.

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