Excitation-contraction coupling in intact frog skeletal muscle fibers injected with mmolar concentrations of fura-2

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ABSTRACT Experiments were carried out to test the hypothesis that mM concentrations of fura-2, a high-affinity Ca^{2+} buffer, inhibit the release of Ca^{2+} from the sarcoplasmic reticulum (SR) of skeletal muscle fibers. Intact twitch fibers from frog muscle, stretched to a long sarcomere length and pressure-injected with fura-2, were activated by an action potential. Fura-2's absorbance and fluorescence signals were measured at different distances from the site of fura-2 injection; thus, the myoplasmic free Ca^{2+} transient ($\Delta[Ca^{2+}]$) and the amount and rate of SR Ca^{2+} release could be estimated at different myoplasmic concentrations of fura-2 ([fura-2_T]). At [fura-2_T] = 2–3 mM, the amplitude and half-width of $\Delta[Ca^{2+}]$ were reduced to ~25% of the values measured at [fura-2_T] < 0.15 mM, whereas the amount and rate of SR Ca^{2+} release were enhanced by ~50% (n = 5; 16°C). Similar results were observed in experiments carried out at low temperature (n = 2; 8.5–10.5°C). The finding of an enhanced rate of Ca^{2+} release at 2–3 mM [fura-2_T] is opposite to that reported by Jacquemond et al. (Jacquemond, V., L. Csernoch, M. G. Klein, and M. F. Schneider. 1991. *Biophys. J.* 60:867–873) from analogous experiments carried out on cut fibers. In two experiments involving the injection of larger amounts of fura-2, reductions in SR Ca^{2+} release were observed; however, we were unable to decide whether these reductions were due to [fura-2_T] or to some nonspecific effect of the injection itself. These experiments do, however, suggest that if large [fura-2_T] inhibits SR Ca^{2+} release in intact fibers, [fura-2_T] must exceed 6 mM to produce an effect comparable to that reported by Jacquemond et al. in cut fibers. Our clear experimental result that 2–3 mM [fura-2_T] enhances SR Ca^{2+} release supports the proposal that $\Delta[Ca^{2+}]$ triggered by an action potential normally feeds back to inhibit further release of Ca^{2+} from the SR (Baylor, S. M., and S. Hollingworth. 1988. *J. Physiol. [Lond.]*. 403:151–192). Our results provide no support for the hypothesis that Ca^{2+}-induced Ca^{2+} release plays a significant role in excitation–contraction coupling in amphibian skeletal muscle.

INTRODUCTION

In a vertebrate skeletal muscle cell, depolarization of the transverse-tubular (T-tubular) membranes triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR); as a result, the myoplasmic Ca^{2+} concentration increases and activation of the myofilaments ensues. From experiments with Ca^{2+}-indicator dyes, the estimated quantity of Ca^{2+} released from the SR in response to a single action potential is ~0.3 mM if referred to the myoplasmic water volume, and the estimated half-width of the release event is brief, ~2–3 ms at 16–18°C (Baylor et al., 1983; Maylie et al., 1987*b*; Baylor and Hollingworth, 1988). The molecular mechanism, however, whereby T-tubular depolarization initiates SR Ca^{2+} release remains uncertain.

A possible mechanism suggested from work on skinned fibers (Endo et al., 1970; Ford and Podolsky, 1970; Stephenson, 1978; Fabiato, 1985) is that the Ca^{2+} ion itself may either initiate or amplify the release process ("Ca^{2+}-induced Ca^{2+} release"). Other work on skinned fibers (Endo, 1981) and SR vesicles (Ikemoto et al., 1985; Meissner et al., 1986) implicates both activating and inhibitory effects of Ca^{2+} on the release process. More recently, studies of individual SR Ca^{2+}-release channels incorporated into bilayers confirm the existence of two effects of Ca^{2+} on the release channel, an activating effect at free Ca^{2+} concentrations in the range 10^{-6}–10^{-4} M (Smith et al., 1986; Ma et al., 1988) and an inhibitory effect at larger free Ca^{2+} (Ma et al., 1988). While the exact physiological relevance of these studies from disrupted preparations is unclear, the finding of an activating effect of Ca^{2+} on the release channel itself has heightened interest in the possible role of Ca^{2+} as an intracellular messenger in the excitation–contraction (E-C) coupling process.

Although the flux of Ca^{2+} across the exterior membranes of a fiber cannot be a significant source of messenger Ca^{2+} during a single twitch (Armstrong et al., 1972), it is possible that Ca^{2+} might come from an intracellular source. Recent speculation (Rios and Pizarro, 1988) has focused on the possibility that Ca^{2+}, initially released from the SR by a Ca^{2+}-independent mechanism, may promote additional release, either by further activation of the initially activated channels or by activation of a second set of channels. In the case of adult frog twitch fibers, circumstantial evidence for the existence of a dual control mechanism has come from the finding that two

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distinct types of ryanodine receptors (equivalently, SR Ca^{2+}-release channels; cf. Fleischer et al., 1985; Imagawa et al., 1987; Lai et al., 1988) are found in these fibers; one receptor type cross-reacts with antibodies made to mammalian skeletal but not cardiac ryanodine receptors, whereas the other type displays the reverse antibody cross-reactivity (Lai et al., 1991).

Recent physiological experiments also appear to directly support the existence of a dual control system in frog twitch fibers (Jacquemond et al., 1991). In these experiments, carried out on "cut" fibers mounted in a Vaseline gap chamber, SR Ca^{2+} release was initiated by voltage-clamp depolarization and monitored by optical changes from Ca^{2+} indicator dyes, antipyrylazo III and fura-2, introduced into the myoplasm. Two components of the SR Ca^{2+} release flux (cf. Melzer et al., 1984; Schneider et al., 1987) were described: (*a*) a smaller, noninactivating component thought to be directly dependent on T-tubular depolarization and (*b*) a larger, inactivating component that appeared to be Ca^{2+} dependent, because it was abolished by introduction of BAPTA (1,2-bis[*p*-aminophenoxy]ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid) or fura-2 itself, both high-affinity Ca^{2+}-buffers, into the myoplasm at concentrations greater than ~2 mM.

The finding of Jacquemond et al. (1991) that the peak rate at which Ca^{2+} is released from the SR is greatly reduced when fura-2 is present in myoplasm at mmolar concentrations stands in contrast to earlier studies carried out on intact (as opposed to cut) frog twitch fibers. Baylor and Hollingworth (1988) and Pape et al. (1990) found that the peak release rate is enhanced substantially in the presence of 0.5–1.5 mM fura-2. However, these studies did not report on fura-2's effects at concentrations > 1.5 mM, and the studies of Jacquemond et al. (1991) did not report on the effects of BAPTA or fura-2 at concentrations < 1.5 mM. Because of the qualitatively different conclusions of these reports, and because of the important implications of the findings of Jacquemond et al. (1991) regarding the possibility of Ca^{2+}-induced Ca^{2+} release in E-C coupling, we have carried out new experiments to test the effect of large fura-2 concentrations (>1.5 mM) on SR Ca^{2+} release in intact fibers.

A preliminary account of the findings has been reported in abstract form (Baylor et al., 1992).

METHODS

The methods employed were similar to those described previously (Baylor and Hollingworth, 1988; Pape et al., 1990). Briefly, an intact single fiber was dissected from an iliofibularis or semitendinosus muscle of *Rana temporaria* and mounted on an optical bench apparatus for the measurement of absorbance, fluorescence, and birefringence signals. The fiber was stretched to a long sarcomere length (3.6–4.3 μm) and lowered onto pedestal supports to minimize movement of artifacts in the optical traces. Fiber activity was initiated by an action potential generated by a suprathreshold shock from a pair of extracellular electrodes positioned near the middle of the fiber. Experiments began with the measurement of a fiber's intrinsic birefringence signal ("second component" of Baylor and Oetliker, 1975; reported as $\Delta I/I$, the fractional change in light intensity). Fibers that had birefringence signals of normal amplitude (peak $\Delta I/I$ of at least −0.001) and time course (time to peak, ~8–9 ms at 16°C) were then pressure injected, as described below, with the pentapotassium salt of fura-2 (Grynkiewicz et al., 1985), obtained from Molecular Probes, Inc. (Junction City, OR; lot Nos. 26121 and 26141). BAPTA was not tested because its Ca^{2+}-buffering action cannot be directly quantitated, as can that of fura-2, by measurement of a change in absorbance and/or change in fluorescence.

Solutions and temperature

The Ringer solution contained (mM): 120 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 5 PIPES (piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)), with pH adjusted to 7.1 by NaOH. A Peltier cooling device set the bath temperature at 16–16.5°C for most experiments; in two experiments (noted in Results) bath temperature was lowered to 8.5–10.5°C, the temperature used by Jacquemond et al. (1991).

Microinjections

A risk of damage to the cell is associated with any experiment in which a fiber is impaled with a microelectrode and microinjected. In general, the risk is increased as the amount of fluid injected is increased. Because a first requirement of the present experiments was to attain mmolar concentrations of fura-2 in myoplasm, it was important to use a technique that both permitted relatively large injections and minimized the possibility of injection damage. For this purpose, a micropipette of relatively low resistance (~3–4 MΩ if filled with 3 M KCl) was pulled from thin-walled capillary tubing (TW150F; WP Instruments, Inc., Sarasota, FL) and filled with a solution of 50 mM K_5fura-2 and 15 mM $CaCl_2$ in distilled water. Pressure (4–10 psi) was applied to the pipette via a Pico-Spritzer II unit (General Valve Corp., Fairfield, NJ); after each impalement the pressure was adjusted so as to inject fura-2 steadily and fairly rapidly, while at the same time avoiding obvious swelling of the fiber. ("Balloon" injections, in which the surface membrane at the injection site became visibly raised due to a large injected volume, were specifically avoided as they were invariably associated with structural alterations and functional impairment of the fiber; cf. next paragraph). The injection itself usually took 2–4 min and was monitored visually under fluorescence optics (excitation filter, 410 nm; emission filter, >480 nm). In nearly all experiments, the injection was completed after a single impalement of the fiber; in one experiment, noted in the legend of Fig. 2, two injections were carried out, at impalement sites separated by ~200 μm.

Selection criteria for a meaningful experiment

If, after the injection of fura-2, the sarcoplasmic reticulum releases a smaller amount of Ca^{2+} in response to electrical stimulation, two general explanations are possible. Either fura-2 has had some specific effect on SR Ca^{2+} release or there has been some damage to the fiber due to the injection process itself. To distinguish these cases, several criteria, both structural and functional, were used as indicators of injection damage. If the fiber did not satisfy all criteria, it was not included in the analysis, on the grounds that any observed effects were likely to reflect nonspecific injection damage rather than a specific fura-2 effect, e.g., one related to fura-2's ability to buffer Ca^{2+}. These criteria were as follows: (*a*) A striation spacing unaltered by the injection process. In some experiments a localized increase in striation spacing, up to 6–7 μm, was seen within a few tens of microns of the injection site. Such increases occurred when the pipette concentration of fura-2 was significantly greater than 50 mM or when balloon injections (cf. preceding paragraph) were carried out at 50 mM fura-2. The presence of such long striation spacings was invariably associated with a local failure of

SR Ca^{2+} release. (*b*) Absence of a significant bleb or tear of the membrane at the injection site, attributable to the electrode impalement per se. (*c*) Presence of a free Ca^{2+} transient of normal amplitude (see Results) measured from a fiber region remote from the injection site, where the myoplasmic concentration of Ca^{2+}-free fura-2 (denoted [fura-2]) was "non-buffering," i.e., <0.1 mM (cf. Baylor and Hollingworth, 1988). (iv) Absence of detectable structural abnormalities when the fiber was viewed under the dissecting microscope at the end of the experiment. Even with these selection criteria, the possibility cannot be excluded that some aspects of the measurements in this report might still reflect effects of injection damage (cf. Figs. 3 and 4 in Results and related discussion).

The molar ratio of $CaCl_2$ to fura-2 in the injection pipette, 0.3, was chosen to minimize any changes in resting myoplasmic free $[Ca^{2+}]$ due to the injected fura-2. Although resting $[Ca^{2+}]$ was not measured in the experiments, the report of Lee et al. (1991) indicates that, in intact amphibian fibers, the fraction of myoplasmic fura-2 in the Ca^{2+}-bound form at rest is probably ~0.3. This value is close to the 0.3–0.5 level thought to apply to fura-2 in cut frog fibers (Klein et al., 1988; Jacquemond et al., 1991).

Optical measurements

Fluorescence

Fluorescence measurements began as soon as possible (~30–60 s) after completion of the fura-2 injection. To minimize any effects due to gradients in the fura-2 concentration within the measurement region, the illuminating field was restricted to a short length of fiber (~60 μm) by means of a slit positioned in the light path and oriented transversely to the fiber axis. With this arrangement, the concentrations of fura-2 at the two edges of the measurement region were within 5–15% of the spatially averaged fura-2 concentration (for those measurement regions that contained strongly buffering concentrations of fura-2, i.e., >1.5 mM). Fura-2 fluorescence was excited with a 410 ± 20-nm interference filter (410 RDF filter; Omega Optical Co., Brattleborough, VT) positioned in the light path between the tungsten–halogen source and the fiber; emission wavelengths > 480 nm were selected for measurement by means of a barrier filter (480 REF filter; Omega Optical Co.) placed between the fiber and the silicon photodiode (UV100; EG&G, Inc., Salem, MA) used to monitor light intensity. In each measurement, a small fraction of the total light intensity was due to incident radiation that was not blocked by the barrier filter. This nonfluorescence component of intensity was recorded in each experiment from a fiber region not containing fura-2 and subtracted from the measured intensity level to obtain the fura-2–related component (denoted F).

Each measurement of fura-2 fluorescence began with a recording of the resting F and was followed immediately by a recording (500 points sampled at 2.5 kHz) of the change in fluorescence intensity (ΔF) resulting from action potential stimulation. The initial fluorescence measurements were made at or within 100 μm of the site of fura-2 injection, where F (and therefore [fura-2]) was large. Additional F and ΔF measurements were then made from other myoplasmic locations by translation of the fiber, in steps of ~100–150 μm, to recording sites progressively more remote from the injection site, where [fura-2] was smaller. When the measurement region was ~400–600 μm from the injection site, [fura-2] was sufficiently small (~0.1 mM) that the noise level in the ΔF trace became limiting in the calculation of $\Delta[Ca^{2+}]$ (see below). The direction of the fiber translation was then reversed and bracketing measurements were made as the recording location was moved back towards the injection site. Thus, within 5–10 min after completion of the injection, 6–10 measurements of F and ΔF were made from fiber regions containing a wide range of [fura-2].

Absorbance

To estimate [fura-2] at the various fiber locations, one particular fiber region (called the "calibration region") was selected for measurement of absorbance changes during activity (ΔA) as well as fluorescence (F and ΔF). The calibration region was usually located 100–200 μm from the injection site, where the F, ΔF, and ΔA signals were large and readily resolved. For measurement of ΔA, a spot of light, the diameter of which was less than the fiber diameter, was focused on the fiber, and fractional changes in transmitted light intensity ($\Delta I/I$) in response to stimulation were recorded at two wavelengths: 420 ± 15 nm, a wavelength where both a fura-2–related change and a fiber-intrinsic change are recorded; and 480 ± 15 nm, a wavelength where only the fiber-intrinsic change is recorded. The intrinsic component was then removed from the 420-nm trace by subtraction of the 480-nm measurement scaled by the factor $(480/420)^{1.6}$ (Hollingworth and Baylor, 1990). The fura-2–related $\Delta I/I$ was converted to ΔA by the relationship

$$\Delta A = -(1/\log_e 10)(\Delta I/I). \quad (1)$$

ΔA was in turn converted to the concentration of Ca^{2+}–fura-2 complex formed during activity (Δ[Cafura-2]) by means of Beer's law and the change in fura-2's effective extinction coefficient at 420 nm (see Baylor and Hollingworth, 1988).

Extension of the calibration

In the absence of any errors due to the "inner filter effect" (i.e., nonlinearities in fluorescence intensity due to fura-2's absorbance of either the incident or emitted radiation; Cantor and Schimmel, 1980), [fura-2] at the calibration region can be calculated from the equation

$$[\text{fura-2}] = -\Delta[\text{Cafura-2}] \times (F/\Delta F) \quad (2)$$

(Baylor and Hollingworth, 1988). Similarly, [fura-2'], the concentration of Ca^{2+}-free fura-2 at another fiber location (or at the same fiber location but a different time) and Δ[Cafura-2'], the change in concentration of Ca^{2+}–fura-2 complex at the second fiber location (or time), can be estimated from the fluorescence measurements, F' and $\Delta F'$, at the new location (or time) by means of the equations

$$[\text{fura-2}'] = [\text{fura-2}] \times (F'/F) \quad (3)$$

and

$$\Delta[\text{Cafura-2}'] = [\text{fura-2}] \times (\Delta F'/F). \quad (4)$$

Eqs. 2–4 follow because, at the excitation wavelengths used, the fluorescence (and absorbance) of the Ca^{2+}-bound form of fura-2 is negligible in comparison with that of the Ca^{2+}-free form of the compound; hence [fura-2], [fura-2'], and Δ[Cafura-2'] are, in the absence of inner-filter-effect errors, linearly related to F, F', and $\Delta F'$, respectively. Because of the inner filter effect, however, which becomes significant at millimolar [fura-2], a straightforward use of Eqs. 2–4 leads to some errors; for example, with an 80-μm-diam fiber and a calibration region located 200 μm from the fura-2 injection site, [fura-2'] at the injection site is typically overestimated by 5–10%, whereas far from the injection site (400–600 μm) [fura-2'] is underestimated by 1–4%. Eq. 5 of Baylor et al. (1981) provides a way to estimate and correct for errors due to the inner filter effect (cf. Konishi et al., 1991); this correction has been applied routinely to all estimates of fura-2 concentrations given in Results.

Calculation of $[\text{fura-2}_T]$ and Δf_{CaD}

Given [fura-2] as calculated above (including the correction for the inner filter effect), the total myoplasmic fura-2 concentration (i.e., both Ca^{2+}-free and Ca^{2+}-bound; denoted $[\text{fura-2}_T]$) can be calculated from

$$[\text{fura-2}_T] = [\text{fura-2}]/(1 - f_{CaD}), \quad (5)$$

where f_{CaD} is the fraction of $[\text{fura-2}_T]$ in the Ca^{2+}-bound form at rest, assumed to be 0.3 for these experiments (see above). The fraction of $[\text{fura-2}_T]$ that changed to the Ca^{2+}-bound form during activity, Δf_{CaD}, is then given by

$$\Delta f_{CaD} = \Delta[\text{Cafura-2}]/[\text{fura-2}_T], \quad (6)$$

where Δ[Cafura-2] is calculated as described above (including the correction for the inner-filter effect).

Calculation of $\Delta[Ca^{2+}]$

Due to kinetic delays in the reaction of fura-2 with Ca^{2+}, the waveform of Δf_{CaD} lags substantially that of $\Delta[Ca^{2+}]$ itself (Baylor and Hollingworth, 1988; Klein et al., 1988). However, as shown in Pape et al. (1990), a reasonably well resolved $\Delta[Ca^{2+}]$ can be calculated from Δf_{CaD} and f_{CaD}, given k_{+1} and k_{-1}, the effective forward and backward rate constants for Ca^{2+}'s reaction with fura-2, assumed to be a single-site reaction of 1:1 stoichiometry. In experiments involving simultaneous recordings with antipyrylazo III and fura-2, Baylor and Hollingworth (1988) determined effective rates at 16°C of 0.25×10^8 $M^{-1}s^{-1}$ for k_{+1} and 23/s for k_{-1} under the assumption that f_{CaD} (a quantity not measured directly in their experiments) was 0.06. On the other hand, if f_{CaD} was 0.3 (cf. Lee et al., 1991), the measurements of Baylor and Hollingworth (1988) imply (unpublished observations) that k_{+1} = 0.25×10^8 $M^{-1}s^{-1}$ and k_{-1} = 17/s. The $\Delta[Ca^{2+}]$ data shown in Results are equivalently derived by use of either of these two sets of values for k_{+1} and k_{-1}, given the corresponding assumption for f_{CaD} (0.06 or 0.3, respectively) in Eqs. 5 and 6. (Note: the value of k_{+1} given above assumes that the amplitude of $\Delta[Ca^{2+}]$, as obtained from the antipyrylazo III signal by means of the in vitro calibration constants of Hollingworth et al. [1987], is erroneously small by a factor of 4. A scaling error in $\Delta[Ca^{2+}]$ of at least fourfold is likely [Maylie et al., 1987*b*; Baylor and Hollingworth, 1988; Konishi et al., 1991] and is attributed to an alteration of the properties of antipyrylazo III that arises because the indicator binds heavily to intracellular constituents. The specific assumption of a fourfold error in $\Delta[Ca^{2+}]$ was made here so that the results of the model calculations described in the next section could be directly compared with those given in Baylor and Hollingworth [1988]. The principal conclusions described in Results do not depend strongly on the exact value assumed for the scaling error in $\Delta[Ca^{2+}]$.)

Modeling of SR Ca^{2+} release

Given $\Delta[Ca^{2+}]$, the kinetic model (model 2) of Baylor et al. (1983), modified slightly as described in Baylor and Hollingworth (1988), was used to estimate the movement of Ca^{2+} between the SR and the myoplasm in response to electrical stimulation. Of relevance to this paper are the model estimates of the amount of Ca^{2+} released from the SR (denoted $\Delta[Ca_T]$, with units referred to the Ca^{2+} concentration increase in the myoplasmic water volume; cf. Baylor et al., 1983) and the rate of Ca^{2+} release from the SR into myoplasm (calculated as the time derivative of $\Delta[Ca_T]$ and denoted $(d/dt)\Delta[Ca_T]$).

Assumptions for quantitation of the cold-temperature experiments

Two experiments described in Results were carried out at colder temperatures (8.5–10.5°C). These experiments were analyzed by the method described in the preceding paragraph but with reduced values for the reaction rates of Ca^{2+} and Mg^{2+} with their binding sites.

Fura-2

Calculations of $\Delta[Ca^{2+}]$ from Δf_{CaD} were carried out under two different assumptions regarding the values of k_{+1} and k_{-1} for the Ca^{2+}–fura-2 reaction; in both cases, the values used correspond to the assumption that f_{CaD} = 0.3 in Eqs. 5 and 6.

(*a*) $k_{+1} = 0.375 \times 10^8$ $M^{-1}s^{-1}$ and k_{-1} = 12/s (with K_D = 0.32 μM). These values are taken from Klein et al. (1988), who obtained estimates from cut fiber experiments at 8–10°C by a method very similar to that used by Baylor and Hollingworth (1988) on intact fibers at 16°C; however, the value given by Klein et al. for k_{+1} (1.5×10^8 $M^{-1}s^{-1}$) has been reduced by a factor of 4 to correct for the error in the amplitude of $\Delta[Ca^{2+}]$ as directly calibrated from the antipyrylazo III signal (cf. discussion above).

(*b*) $k_{+1} = 0.175 \times 10^8$ $M^{-1}s^{-1}$ and k_{-1} = 12/s (K_D = 0.69 μM). These values follow from those given by Baylor and Hollingworth (1988) for 16°C after an adjustment for a temperature change to 9°C. The basis for the adjustment was to assume that (*i*) the value of k_{+1} is governed by a Q_{10} of 2, the value found by Kao and Tsien (1988) for the related Ca^{2+} indicator azo-1, and (*ii*) the same Q_{10} applies to k_{-1}, because fura-2's K_D changes little with temperature (Uto et al., 1991).

Troponin

The reaction rates assumed to apply to the Ca^{2+}–troponin reaction at 16°C, $k_{+1} = 0.89 \times 10^8$ $M^{-1}s^{-1}$ and k_{-1} = 115/s (Baylor and Hollingworth, 1988), were lowered to 0.77×10^8 $M^{-1}s^{-1}$ and 65/s, respectively. The factor that relates the values of k_{+1}, 0.87, is approximately the factor that relates the viscosity of water at the two temperatures, whereas the factor that relates the values of k_{-1}, 0.57, was obtained under the assumption that in frog muscle troponin's K_D for Ca^{2+} is reduced by a factor of 0.65 if temperature is lowered from 16 to 9°C (Godt and Lindley, 1982).

Parvalbumin

Two sets of rates were assumed for the reactions of Ca^{2+} and Mg^{2+} with parvalbumin at 9°C. (*a*) for Ca^{2+}: $k_{+1} = 0.77 \times 10^8$ $M^{-1}s^{-1}$ and k_{-1} = 0.3/s; and for Mg^{2+}: $k_{+1} = 2.0 \times 10^4$ $M^{-1}s^{-1}$ and k_{-1} = 1.8/s. These values follow from the rates given in Baylor et al. (1983) for 16°C under the assumption that a Q_{10} of 2 applies to all rates; a Q_{10} of 2 is consistent with the temperature dependence of the k_{-1}'s as measured by Hou et al. (1991). (*b*) for Ca^{2+}: $k_{+1} = 3.86 \times 10^8$ $M^{-1}s^{-1}$ and k_{-1} = 0.3/s; and for Mg^{2+}: $k_{+1} = 1.0 \times 10^5$ $M^{-1}s^{-1}$ and k_{-1} = 1.8/s. The values of k_{-1} are the same as in set (*a*) above, whereas the values of k_{+1} assume that K_D is governed by a Q_{10} of 10 in this temperature range (Ogawa and Tanokura, 1986).

Resting Ca^{2+}

Two assumptions were made regarding the level of resting $[Ca^{2+}]$: (*a*) $[Ca^{2+}]$ = 0.02 μM (Baylor et al., 1983; Baylor and Hollingworth, 1988), and (*b*) $[Ca^{2+}]$ = the value required to give f_{CaD} = 0.3 for fura-2 {either $[Ca^{2+}]$ = 0.14 μM or $[Ca^{2+}]$ = 0.29 μM, depending on which of the assumptions was made concerning fura-2's reaction kinetics ([*a*] or [*b*], respectively, given above)}.

The two different assumptions for the fura-2 kinetics, the two different assumptions for the parvalbumin kinetics, and the two different assumptions for resting $[Ca^{2+}]$ give, in total, eight different ways to estimate the SR Ca^{2+} release rates for each measurement of F and ΔF. The use of these eight ways provided a reasonable range of models with which to interpret the results of the cold-temperature experiments.

RESULTS

I. Effects of [fura-2_T] in the 0–3-mM range

As described in Methods, a single fiber injected with fura-2 was activated by action potential stimulation; and fluorescence signals (F and ΔF) were measured from a number of fiber regions, each of which contained a different myoplasmic concentration of fura-2. The superimposed pairs of traces in Fig. 1 compare results from two such regions: the dotted traces reflect events 400 μm from the injection site, where [fura-2_T] was relatively small (0.14 mM), whereas the continuous traces reflect events 100 μm from the injection site, where [fura-2_T] was large (2.26 mM). Four types of traces are shown: (*a*) Δ[Cafura-2], the change in concentration of Ca^{2+}-

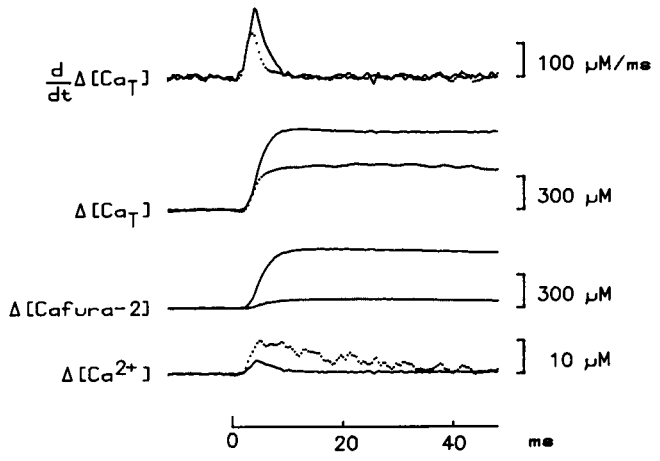

FIGURE 1 Comparison of Ca^{2+}-related signals triggered by a single action potential from two regions of the same fiber that contained quite different myoplasmic concentrations of fura-2. Zero time marks the moment of the external shock. The dotted traces were recorded 400 μm from the site of fura-2 injection, where [fura-2_T] was 0.14 mM, whereas the continuous traces were recorded 100 μm from the injection site, where [fura-2_T] was 2.26 mM. At [fura-2_T] = 2.26 mM, the peak of the free Ca^{2+} transient ($\Delta[Ca^{2+}]$) was reduced in comparison with that recorded at [fura-2_T] = 0.14 mM, whereas substantial increases occurred in the peaks of the change in concentration of Ca^{2+}-bound fura-2 (Δ[Cafura-2]), the change in the total myoplasmic concentration of Ca^{2+} due to SR Ca^{2+} release ($\Delta[Ca_T]$), and the rate at which Ca^{2+} was released from the SR ($(d/dt)\Delta[Ca_T]$). Fiber no., 071791.1; fiber diameter, 78 μm; temperature, 16.0°C.

bound fura-2 (which is directly related to the measurement of ΔF); (*b*) $\Delta[Ca^{2+}]$, the myoplasmic free Ca^{2+} transient, calculated from the Δ[Cafura-2] waveform after conversion of the latter to Δf_{CaD} units and application of the kinetic correction described in Methods; (*c*) $\Delta[Ca_T]$, the modeled estimate related to the amount of Ca^{2+} released from the SR, obtained as the summed change in the Ca^{2+} concentration in four myoplasmic pools: free, bound by fura-2, bound by troponin, and bound by parvalbumin; and (*d*) $(d/dt)\Delta[Ca_T]$, the modeled estimate of the rate of SR Ca^{2+} release (in units of the rate of change of $\Delta[Ca_T]$).

A comparison of the traces in Fig. 1 reveals the following differences for [fura-2_T] at a strongly buffering concentration (2.26 mM) compared with a slightly buffering concentration (0.14 mM): (*a*) the concentration of Ca^{2+}-bound fura-2 was greatly increased (from 80 to 540 μM); (*b*) the peak amplitude and half-width of $\Delta[Ca^{2+}]$ were decreased substantially (from 10 to 4 μM, and from ~15 to 4 ms, respectively); (*c*) the rise in the total myoplasmic concentration of Ca^{2+} due to SR release was increased by ~90% (from 380 to 720 μM); (*d*) the peak rate at which Ca^{2+} was released from the SR was increased by ~50% (from 130 to 200 μM/ms).

In Fig. 2 the ordinates of the panels show collected results of four key measurements made from traces of the type shown in Fig. 1, not only for the experiment of Fig. 1 (*triangles*) but for four other experiments as well (each indicated by a different symbol). The abscissas for the panels in Fig. 2 are all the same, namely, [fura-2_T]. In each panel, a dashed line is also shown, for which the intercept on the ordinate corresponds to the average value of the measurement as observed in previous experiments carried out with antipyrylazo III at nonbuffering concentrations (Baylor and Hollingworth, 1988); the zero slope of these lines corresponds to the measurements being independent of [fura-2_T]. In the case of all four measurements, however, a clear dependence on [fura-2_T] in the range 0–3 mM is seen: the amplitudes of both $\Delta[Ca_T]$ (*A*) and $(d/dt)\Delta[Ca_T]$ (*B*) increase, whereas the amplitude of $\Delta[Ca^{2+}]$ (*C*) and the half-width of $\Delta[Ca^{2+}]$ (*D*) decrease. The major fraction of these changes is seen for [fura-2_T] between 0 and 1 mM, with relatively little additional change seen for [fura-2_T] between 1 and 3 mM.

We attribute these changes to effects of [fura-2_T] because they were observed in fibers that (*a*) by the selection criteria described in Methods did not experience obvious fiber damage as a result of the injection itself and (*b*) displayed neither of the functional alterations described in part II of Results that are thought to be indicative of some less obvious type of fiber damage. For the five experiments in Fig. 2, the average value of the amplitude of $(d/dt)\Delta[Ca_T]$ (*B*) was 179 ± 14 μM/ms (±SEM) for [fura-2_T] = 2–3 mM compared with the value 120 ± 6 μM/ms for [fura-2_T] < 0.15 mM. The finding that the peak rate of SR Ca^{2+} release is increased substantially at higher [fura-2_T] is opposite to the result described in cut fibers, where the peak observed at [fura-2_T] = 2–3 mM was only one-third to one-fourth that observed in the same fibers at [fura-2_T] = 0.05 mM (Jacquemond et al., 1991).

Results at colder temperatures

To test whether the qualitative difference between our results, obtained at 16°C, and those of Jacquemond et al. (1991), obtained at 8–10°C, might be related to the temperature of the experiments, we attempted additional experiments, analogous to those of Figs. 1 and 2, at colder temperatures. Two such experiments were successful, one at 8.5°C, in which the maximum value of [fura-2_T] was 2.9 mM, and one at 10.5°C, in which the maximum value was 2.1 mM. In both experiments and under all eight ways of estimating SR Ca^{2+} release (cf. Methods), peak values of $\Delta[Ca_T]$ and $(d/dt)\Delta[Ca_T]$ increased with increasing [fura-2_T], as was the case in Fig. 2. For the two experiments, the averaged results at [fura-2_T] > 2 mM, when compared with those at [fura-2_T] < 0.13 mM, showed increases that ranged between 25 and 69% for $\Delta[Ca_T]$ and between 16 and 81% for $(d/dt)\Delta[Ca_T]$, with the range in the numbers reflecting the eight ways used to obtain the model estimates. We therefore conclude that the difference between our results and those of Jacquemond et al. (1991) concerning the effect of 2–3

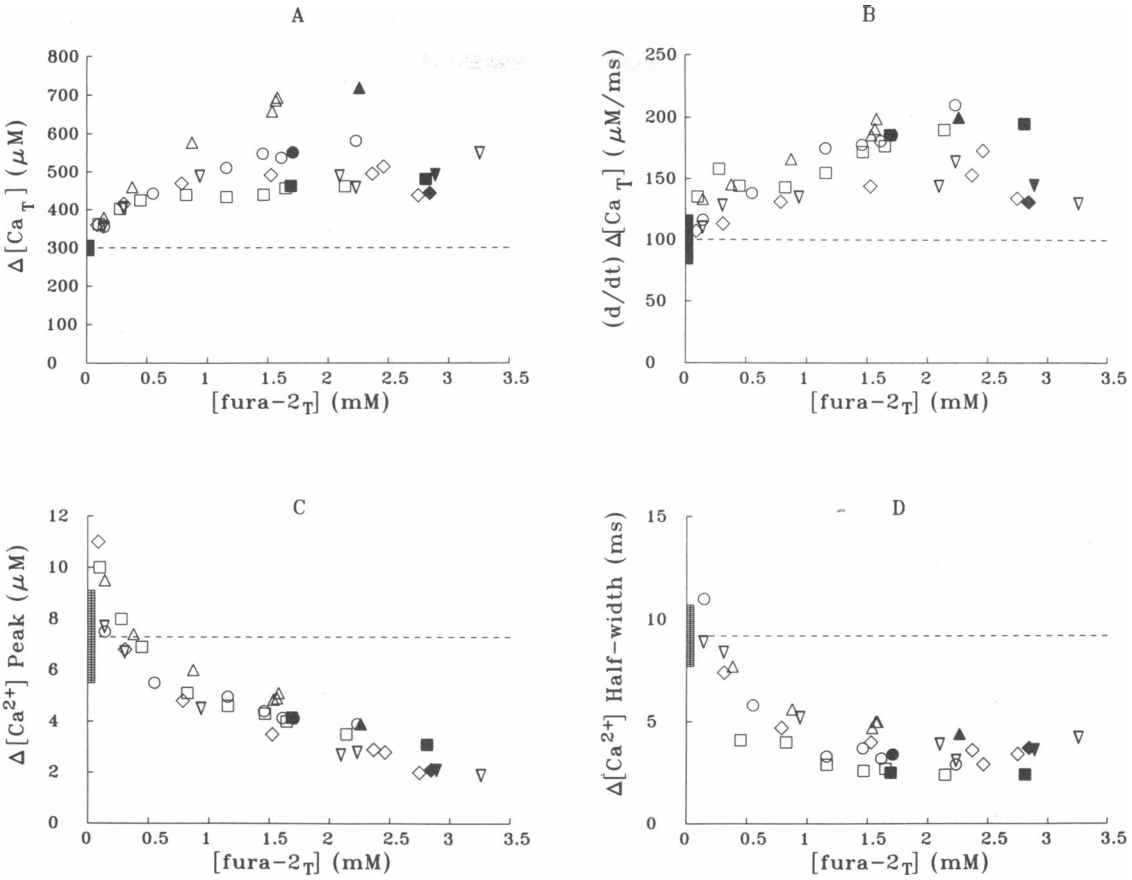

FIGURE 2 Ca^{2+}-related signals observed in response to an action potential (*ordinate*) plotted vs. the total concentration of fura-2 in myoplasm (*abscissa*); the ordinate values were measured from traces of the type shown in Fig. 1. Each symbol type represents values obtained from a single experiment (*circles*, fiber 071591.2; *squares*, 071591.4; *triangles*, 071791.1; *diamonds*, 071791.4; *inverted triangles*, 071591.3). The filled symbols identify measurements taken at the site of fura-2 injection, where the possibility of injection damage was the greatest. For the five experiments, fiber diameters varied between 74 and 88 μm, and temperatures between 16.0 and 16.2°C. The dashed lines and vertical bars represent means and ±1 SD units, respectively, for the measurements as determined in previous experiments in the absence of fura-2 (Baylor and Hollingworth, 1988). (*A*) The increase in total myoplasmic Ca^{2+} concentration, measured 9–10 ms after stimulation. (*B*) The peak rate of SR Ca^{2+} release. {Note: these values occurred 2.5–4.4 ms after stimulation, except for the measurements corresponding to the two largest values of [fura-2_T] in fiber 071591.3, for which the peaks occurred 5.2 ms after stimulation ([fura-2_T] = 2.89 mM) and 6.4 ms after stimulation ([fura-2_T] = 3.26 mM). Because fiber 071591.3 was subjected to two injections of fura-2 and the measurements corresponding to the two largest [fura-2_T]'s were made at or within 100 μm of the site of the larger of the two injections, the somewhat slower times to peak observed for these two measurements could reflect injection damage.} (*C*) The peak value of the myoplasmic free calcium concentration. (*D*) The half-width of the myoplasmic free calcium concentration. (Note: for four of the six measurements made at [fura-2_T] < 0.2 mM, the half-width of $\Delta[Ca^{2+}]$ could not be reliably determined; thus points corresponding to these measurements do not appear in *D*)

mM [fura-2_T] on SR Ca^{2+} release is not likely to reflect an effect of temperature on the release process.

(Note: averaged over the two experiments, our values of $\Delta[Ca_T]$ ranged from 239 to 376 μM at [fura-2_T] < 0.13 mM and from 385 to 524 μM at [fura-2_T] > 2 mM, whereas the values of $(d/dt)\Delta[Ca_T]$ ranged from 49 to 97 μM/ms for [fura-2_T] < 0.13 mM and from 87 to 112 μM/ms at [fura-2_T] > 2 mM. The values 87–112 μM/ms are an order of magnitude larger than the release rates reported by Jacquemond et al. (1991) for cut fibers containing 2–3 mM [fura-2_T] or [$BAPTA_T$] and activated by a 200-ms voltage-clamp step to +20 mV. The basic difference in the experimental measurements is that we observe a Δ[Cafura-2] signal that rises rapidly after a single action potential, whereas Jacquemond et al. observe a Δ[Cafura-2] or Δ[CaBAPTA] signal that, although it rises eventually to a larger peak value, has a maximum rate of rise that is an order of magnitude smaller.)

II. Results of experiments at [fura-2_T] > 3 mM

It is also possible that the difference between our results and those of Jacquemond et al. (1991) reflects the difference in the experimental preparations: intact fibers vs. cut fibers, respectively. The physiological state of cut fibers is reported to change progressively with time following cutting (Maylie et al., 1987*a*). Moreover, in cut fibers the voltage dependence of muscle charge movement (a signal that is closely related to SR Ca^{2+} release)

is often less steep than that observed in intact fibers, with considerable variation in steepness observed from one laboratory to another (cf. Table 7 of Hui and Chandler, 1990). The reason for this variation is not known but may be related to the method of preparation of the fibers. Given these findings, one might hypothesize that all fibers, both intact and cut, would show reductions in the amount and rate of SR Ca^{2+} release if [fura-2_T] were large enough, but that the [fura-2_T] required to produce this effect happens to be smaller for the cut fibers of Jacquemond et al. (1991) than for our intact fibers. Thus, it might be possible to demonstrate a reduction in Ca^{2+} release in intact fibers if [fura-2_T] could be made larger than the maximum levels achieved for the experiments of Figs. 1 and 2.

To investigate this possibility, we attempted experiments at 16°C that involved larger fura-2 injections. Although these experiments were technically difficult, two experiments were completed that satisfied the selection criteria described in Methods. The principal findings from these experiments are shown in Figs.3 and 4.

The upper panels of Fig. 3 show results from one of the fibers, for which the largest value of [fura-2_T] was 5.5 mM. The data for both $\Delta[Ca_T]$ (*A*) and $(d/dt)\Delta[Ca_T]$ (*B*) appear to show a biphasic dependence on [fura-2_T], with an increase in amplitude for [fura-2_T] between 0 and 3 mM (as for the fibers of Fig. 2) and a decrease in amplitude at higher [fura-2_T]. The latter effect is in the direction expected from the report of Jacquemond et al. (1991). A close inspection of the data, however, reveals that some factor other than [fura-2_T] may underlie the decrease in amplitude at higher [fura-2_T]. This is apparent from a comparison of the filled triangles, which show measurements taken at the injection site, and the open triangles, which show measurements taken at other sites, located ≥ 100 μm from the injection site. At similar values of [fura-2_T] (3–5 mM), SR Ca^{2+} release at the injection site was clearly less than that away from the injection site. The most likely explanation for this difference is that there was local damage to the fiber resulting from the injection. Given this evidence for injection damage, it is not possible to decide whether the negative slope in the relationship between the filled triangle data and [fura-2_T] reflects an effect of [fura-2_T] per se or a time-dependent recovery from the injection damage. (Note: at the injection site, [fura-2_T] decreases monotonically with time after injection, due to diffusion of fura-2 away from the injection site.) Similarly, it is possible that the negative slope in the relationship between the open triangle data and the values of [fura-2_T] exceeding 3 mM was due to injection-related damage. In particular, injection damage might affect measurements made only 100 μm from the injection site (cf. *downward arrows* in Fig. 3, *A* and *B*) in a manner similar to the injection site itself, even though the measurements from the more remote fiber locations, where [fura-2_T] was <3 mM, reveal the pattern expected from Fig. 2. Thus, a convincing argument cannot be made from this experiment that the reduction in SR Ca^{2+} release apparent at [fura-2_T] > 3 mM was in fact due to [fura-2_T].

Fig. 3, *C* and *D*, shows analogous results from the second experiment, in which the maximum value of [fura-2_T] was 8.6 mM. These data are also biphasic, with a prominent reduction in SR Ca^{2+} release beginning at [fura-2_T] > 5 mM. An inspection of the temporal waveforms corresponding to these release signals, however, again suggests that this reduction may not be a direct consequence of [fura-2_T]. Fig. 4 compares experimental waveforms for the two measurements taken at the injection site in this fiber (cf. *filled circles,* numbered *1* and *8* in *C* and *D* of Fig. 3). Within each superimposed pair of traces in Fig. 4, the continuous trace was taken at a smaller value of [fura-2_T] (6.4 mM), whereas the dotted trace was taken at a larger value of [fura-2_T] (8.6 mM). The kinetic waveforms at the larger [fura-2_T] have significantly slower time courses than do those at the smaller [fura-2_T]. For example, on the rising phase of the Δ[Cafura-2] waveforms the time to half peak is 3.6 ms for the continuous trace and 5.2 ms for the dotted trace, whereas the time to peak of the $(d/dt)\Delta[Ca_T]$ waveform is 3.6 ms for the continuous trace and 5.6 ms for the dotted trace. Moreover, an examination of the other measurements in this experiment revealed a correlation between the time courses of the waveforms and the numerical order of the measurements (indicated by the numbers next to the data points in Fig. 3, *C* and *D*), independent of the level of [fura-2_T]. For example, the times to half peak of the Δ[Cafura-2] waveforms corresponding to data points numbered *2* through *6* were 4.8–5.2 ms; i.e., they were similar to the 5.2-ms value of data point *1*, whereas the times to half peak of the waveforms corresponding to data points *7* and *9* were smaller, 3.6 and 4.2 ms, similar to the 3.6-ms value of data point *8*. Thus some factor that was related to the time after injection rather than to the level of [fura-2_T] itself clearly influenced the measurements of this experiment. Again, this factor is most likely some form of injection damage, from which the fiber appears to have recovered, at least partially, with time. Because of the presence of this factor, it is not possible to decide whether the reduction in SR Ca^{2+} release observed at [fura-2_T] > 5 mM was in fact due to [fura-2_T] or to this other factor.

It is of interest to note several other points about the experiment of Fig. 3, *C* and *D*, and Fig. 4. First, because the dramatic reduction in the rate of SR Ca^{2+} release observed for the dotted trace in Fig. 4 was accompanied by a delay in the time course of Ca^{2+} release, the underlying cause for these effects does not appear to be consistent with the mechanism proposed by Jacquemond et al. (1991) to explain how fura-2 may inhibit Ca^{2+} release. According to their theory, the initial rise of the release waveform is controlled by T-tubular voltage, followed secondarily by a Ca^{2+}-dependent component that can be blocked by sufficient [fura-2_T]. Therefore, the initial rise

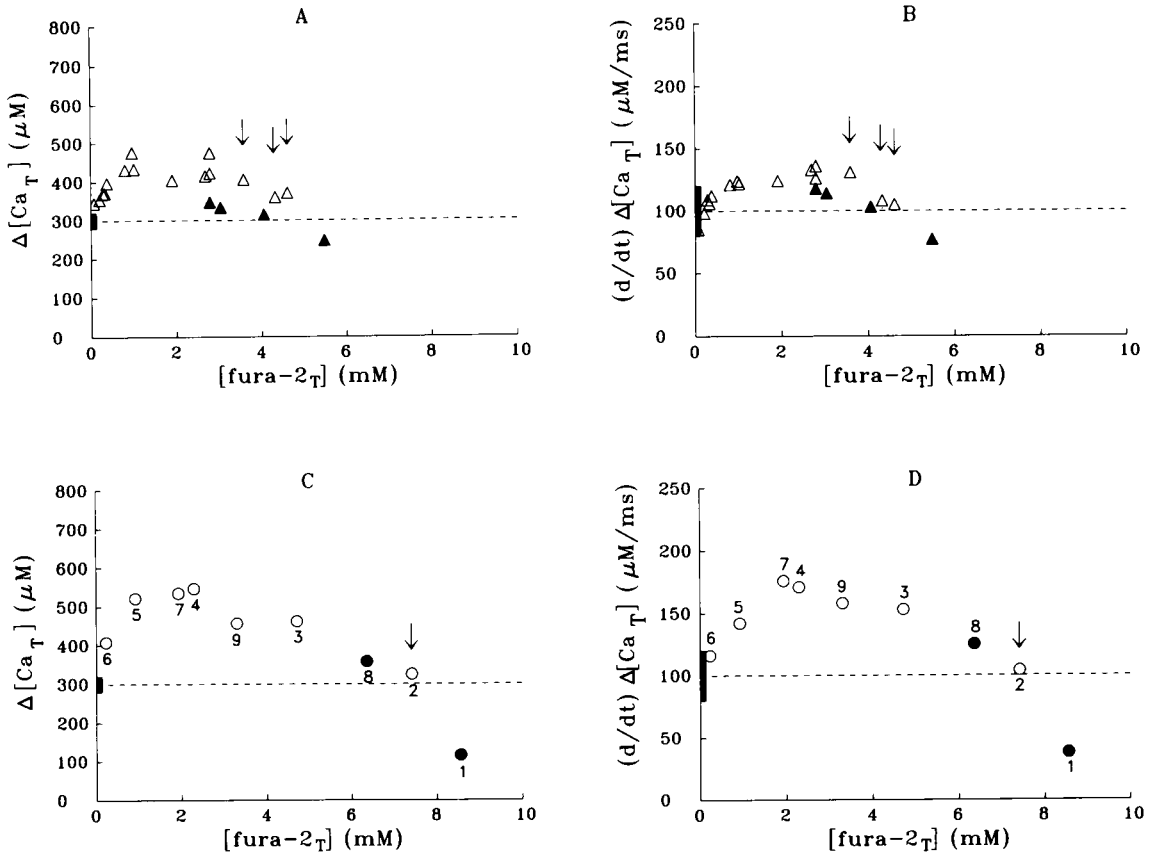

FIGURE 3 The ordinates show Ca^{2+}-related signals plotted vs. the myoplasmic fura-2 concentration for two experiments that involved unusually large injections of fura-2 (fiber diameters, 41–53 μm; temperature, 16.0–16.2°C). Each symbol type represents a single experiment (*triangles*, fiber 072391.3; *circles*, fiber 071791.2). (*A* and *C*) The increase in total myoplasmic Ca^{2+} concentration, measured 9–10 ms after stimulation. (*B* and *D*) The peak rate of SR Ca^{2+} release. (Note: in *B*, peaks occurred between 2.4 and 3.6 ms after stimulation, whereas in *D*, peaks occurred between 3.6 and 5.6 ms; see text.) For fiber 071791.2, the numbers next to the symbols specify the numerical order of the measurements. In all panels, the filled symbols identify measurements taken at the site of fura-2 injection, whereas the downward arrows identify measurements made 100 μm from the injection site; the remaining measurements were made 200 μm or more from the injection site.

of the release waveform should not be delayed by fura-2, as it is in Fig. 4. Thus, independent of whether injection damage is responsible for the reduced amplitude of the Ca^{2+} release signal measured at [fura-2_T] = 8.6 mM in Fig. 4, the presence of a delay in this signal is not consistent with the dual control system proposed by Jacquemond et al. (1991). Second, because the amplitude of data point *8* in Fig. 3 *D* is above the dashed line, the experiment indicates that if [fura-2_T] inhibits SR Ca^{2+} release in intact fibers, [fura-2_T] must exceed 6 mM to produce an effect comparable to that reported for cut fibers (cf. Jacquemond et al., 1991). As a final note, the measurements in Fig. 3, *C* and *D*, may also be influenced by an irreversible, or more slowly reversible, component of injection damage in addition to the reversible kinetic change described above. For example, the finding that in Fig. 3, *C* and *D*, the amplitude of data point *8* (taken at the injection site) is smaller than that of point *9* (taken 200 μm from the injection site) could simply reflect a relatively small component of injection damage localized near the injection site (cf. Fig. 3, *A* and *B*, discussed above).

DISCUSSION

Results, part I ([fura-2_T] 0–3 mM)

Our experiments (Figs. 1 and 2) show that, in intact frog twitch fibers injected with fura-2 and stimulated by an action potential, the myoplasmic free Ca^{2+} transient and the amount and rate of SR Ca^{2+} release depend on the myoplasmic fura-2 concentration. With increasing [fura-2_T], up to 2–3 mM, the amplitude and half-width of $\Delta[Ca^{2+}]$ progressively decrease, to ~20–25% of the values observed at low [fura-2_T] (~0.1 mM); in contrast, the amount and rate of SR Ca^{2+} release increase, to ~150% of the values observed at low [fura-2_T] (Fig. 2). These results confirm and extend the previous findings reported for [fura-2_T] up to 1–1.5 mM (Baylor and Hollingworth, 1988; Pape et al., 1990). In the latter studies, fura-2 was microinjected from a pipette that contained 25 mM fura-2 and no added Ca^{2+}. Thus, the finding that mmolar [fura-2_T] enhances SR Ca^{2+} release in response to an action potential cannot be attributed to an elevation in resting $[Ca^{2+}]$ (and thus a possible overloading of

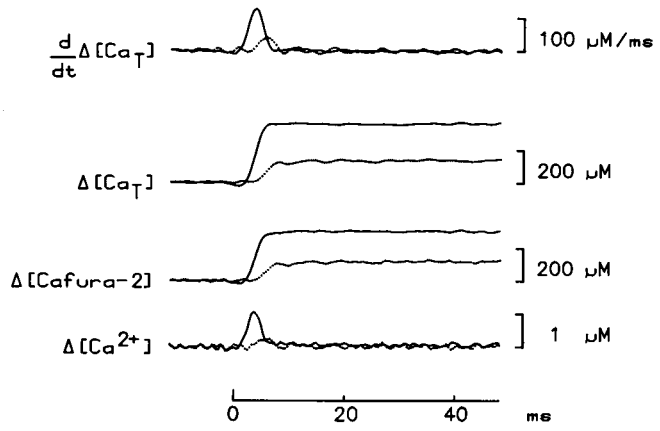

FIGURE 4 As in Fig. 1, this figure compares Ca^{2+}-related signals triggered by a single action potential. Measurements were made at the site of fura-2 injection at two different times: 1 min after completion of the fura-2 injection (*dotted traces*) and 4.5 min after the injection (*continuous traces*); the corresponding values of [fura-2_T] were 8.56 and 6.36 mM, respectively. At the later time, the free Ca^{2+} transient ($\Delta[Ca^{2+}]$) was larger, as was the change in concentration of Ca^{2+}-bound fura-2 (Δ[Cafura-2]), the change in the total myoplasmic concentration of Ca^{2+} due to SR Ca^{2+} release ($\Delta[Ca_T]$), and the rate at which Ca^{2+} was released from the SR ($(d/dt)\Delta[Ca_T]$). To increase the signal-to-noise ratio in the records, all waveforms have been filtered by a 5-point digital smoothing routine (cf. p. 34–35 of Hamming, 1977). Fiber No., 071791.2; fiber diameter, 41 μm; temperature, 16.2°C.

the SR with Ca^{2+}) due to coinjection of Ca^{2+} with the fura-2. (Note: in the present experiments, the molar ratio of Ca^{2+} to fura-2 in the pipette was 0.3; see Methods.) Rather, the results are consistent with the idea that a rise in myoplasmic free $[Ca^{2+}]$ normally inhibits SR Ca^{2+} release (Baylor et al., 1983; Schneider and Simon, 1988; Simon et al., 1991) and that this inhibition is substantial even in response to a single action potential (Baylor and Hollingworth, 1988).

Our finding of an enhancement in the peak rate of SR Ca^{2+} release at 2–3 mM [fura-2_T] is very different from the fourfold reduction in release rate observed in cut fibers activated by voltage-clamp depolarization (Jacquemond et al., 1991). The reason for this striking qualitative difference is unclear. One possibility is that the difference is due to the method of stimulation, a single action potential versus a 200-ms voltage clamp depolarization to +20 mV. The membranes of the transverse tubules presumably charge less rapidly in response to a voltage clamp step to +20 mV than in response to an action potential. If SR Ca^{2+} release involved two opposing Ca^{2+}-dependent mechanisms, one activating and one inhibitory, that occurred with overlapping time courses, then the two methods of stimulation might give qualitatively different results. For example, with action potential stimulation, mmolar fura-2 might reduce $\Delta[Ca^{2+}]$ to a level sufficient to block the inhibitory but not the activating mechanism. In this regard, it may be noted that while $\Delta[Ca^{2+}]$ in Figs. 1 and 2 was reduced substantially in the presence of 2–3 mM [fura-2_T], it was not reduced to the submicromolar levels reported for cut fibers by Jacquemond et al. (1991). Given this possibility, it would be of interest to study the effects of 2–3 mM fura-2 in the intact fiber by a procedure (e.g., the voltage-clamp technique) that permitted a progressive lowering of the rate of SR Ca^{2+} release.

Another possibility is that the cut fibers of Jacquemond et al. (1991) were in some altered physiological state (cf. Results) such that their response in the presence of mmolar fura-2 was basically different from our intact fibers. In this regard it would be of interest to know whether, in response to action potential stimulation, the rate of SR Ca^{2+} release in cut fibers depends on [fura-2_T] in the manner shown in Fig. 2. It would also be of interest to know if, at some level of [fura-2_T] smaller than 2–3 mM, the early peak in SR Ca^{2+} release is blocked in response to a small depolarization but not blocked in response to a large depolarization.

Results, part II ([fura-2_T] > 3 mM)

The experiments described in Figs. 3 and 4 (see also Methods) lead us to conclude that it is difficult to study the effects of [fura-2_T] above 3 mM in the intact fiber by the method of microinjection. Although reductions in SR Ca^{2+} release were observed in two experiments, it is clear that in both experiments effects other than the level of [fura-2_T] were also associated with these reductions. Because these other effects were correlated with proximity to the injection site and/or to the time of injection, they are likely to be a consequence of the injection itself. Unfortunately, the presence of these effects made it impossible to decide whether SR Ca^{2+} release was inhibited in these experiments by [fura-2_T] above 3–4 mM. The experiments do, however, suggest that if very large [fura-2_T] reduces the peak rate of SR Ca^{2+} release in an intact fiber activated by an action potential, [fura-2_T] must exceed 6 mM to produce an inhibitory effect comparable to that seen in cut fibers activated by a voltage-clamp step to +20 mV.

Final conclusion

Although our experiments do not rule out a possible role for Ca^{2+}-induced Ca^{2+} release in E-C coupling, our clear finding of an enhancement of SR Ca^{2+} release at 2–3 mM [fura-2_T] is opposite to the effect expected for Ca^{2+}-induced Ca^{2+} release and therefore does not support this mechanism. It is to be hoped that future experiments will elucidate (*a*) the reason for the qualitatively different effects reported for mmolar [fura-2_T] on Ca^{2+} release in intact and cut fibers and (*b*) whether there is any functional significance for E-C coupling to the finding that amphibian twitch fibers have two types of ryanodine receptor molecules (Lai et al., 1991).

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REFERENCES

Armstrong, C. M., F. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. *Biochem. Biophys. Acta.* 267:605–608.

Baylor, S. M., and S. Hollingworth. 1988. Fura-2 calcium transients in frog skeletal muscle fibres. *J. Physiol. (Lond.).* 403:151–192.

Baylor, S. M., and H. Oetliker. 1975. Birefringence experiments on isolated skeletal muscle fibres suggest a possible signal from the sarcoplasmic reticulum. *Nature (Lond.).* 253:97–101.

Baylor, S. M., W. K., Chandler, and M. W. Marshall. 1981. Studies in skeletal muscle using optical probes of membrane potential. *In* Regulation of Muscle Contraction: Excitation-Contraction Coupling. A. D. Grinnell and Mary A. B. Brazier, editors. Academic Press, New York. 97–130.

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1983. Sarcoplasmic reticulum calcium release in frog skeletal muscle fibres estimated from Arsenazo III calcium transients. *J. Physiol. (Lond.)* 344:625–666.

Baylor, S. M., A. B. Harkins, N. Kurebayashi, M. Konishi, and S. Hollingworth. 1992. Ca^{2+} release from the sarcoplasmic reticulum (SR) of intact frog skeletal muscle fibers containing millimolar concentrations of fura-2. *Biophys. J.* 61:A131. (Abstr.)

Cantor, C. R., and P. R. Schimmel. 1980. Biophysical Chemistry Part II. W. H. Freeman and Company, San Francisco, CA. 443 pp.

Endo, M. 1981. Mechanism of calcium-induced calcium release in the SR membrane. *In* Mechanism of Gated Calcium Transport across Biological Membranes. S. T. Ohnishi and M. Endo, editors. Academic Press, New York. 257–264.

Endo, M., M. Tanaka, and Y. Ogawa. 1970. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibers. *Nature (Lond.).* 228:34–36.

Fabiato, A. 1985. Calcium-induced release of Ca^{2+} from the sarcoplasmic reticulum of skinned fibres from the frog semitendinosus. *Biophys. J.* 47:195*a*. (Abstr.).

Fleischer, S., E. M. Ogunbunmi, M. C. Dixon, and E. A. M. Fleer. 1985. Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 87:7256–7259.

Ford, L. E., and R. J. Podolsky. 1970. Regenerative calcium release within muscle cells. *Science (Wash. DC).* 167:58–59.

Godt, R. E., and B. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J. Gen. Physiol.* 80:279–297.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Hamming, R. W. 1977. Digital Filters. Prentice-Hall, Englewood Cliffs, NJ. 34–35.

Hollingworth, S., and S. M. Baylor. 1990. Changes in Phenol Red absorbance in response to electrical stimulation of frog skeletal muscle fibers. *J. Gen. Physiol.* 96:473–491.

Hollingworth, S., R. W. Aldrich, and S. M. Baylor. 1987. In vitro calibration of the equilibrium reactions of the metallochromic indicator dye Antipyrylazo III with calcium. *Biophys. J.* 51:383–393.

Hou, T.-t., J. D. Johnson, and J. A. Rall. 1991. Parvalbumin content and Ca^{2+} and Mg^{2+} dissociation rates correlated with changes in relaxation rate of frog muscle fibres. *J. Physiol. (Lond.).* 441:285–304.

Hui, C. S., and W. K. Chandler. 1990. Intramembranous charge movement in frog cut twitch fibers mounted in a double vaseline-gap chamber. *J. Gen. Physiol.* 96:257–297.

Ikemoto, N., B. Antoniu, and L. G. Mezaros. 1985. Rapid flow chemical quench studies of calcium release from isolated sarcoplasmic reticulum. *J. Biol. Chem.* 260:14096–14100.

Imagawa, T., J. S. Smith, R. Coronado, and K. P. Campbell. 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+} permeable pore of the calcium release channel. *J. Biol. Chem.* 262:16636–16643.

Jacquemond, V., L. Csernoch, M. G. Klein, and M. F. Schneider. 1991. Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophys. J.* 60:867–873.

Kao, J. P. Y., and R. Y. Tsien. 1988. Ca^{2+} binding kinetics of fura-2 and azo-1 from temperature-jump relaxation measurements. *Biophys. J.* 53:635–639.

Klein, M. G., B. J. Simon, G. Szucs, and M. F. Schneider. 1988. Simultaneous recording of calcium transients in skeletal muscle using high and low affinity calcium indicators. *Biophys. J.* 53:971–988.

Konishi, M., S. Hollingworth, A. B. Harkins, and S. M. Baylor. 1991. Myoplasmic calcium transients in frog skeletal muscle fibers monitored with the fluorescent indicator furaptra. *J. Gen. Physiol.* 97:271–301.

Lai, F. A., H. P. Erickson, E. Rousseau, G.-Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (Lond.).* 331:315–319.

Lai, F. A., Q.-Y. Liu, A. El-Hashem, and G. Meissner. 1991. Immunological evidence for the expression of skeletal- and cardiac-like ryanodine receptor isoforms in amphibian skeletal muscle. *Biophys. J.* 59:63*a*. (Abstr.)

Lee, J. A., H. Westerblad, and D. G. Allen. 1991. Changes in tetanic and resting $[Ca^{2+}]_i$ during fatigue and recovery of single muscle fibres from *Xenopus laevis*. *J. Physiol. (Lond.).* 433:307–326.

Ma, J., M. Fill, C. M. Knudson, K. P. Campbell, and R. Coronado. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science (Wash. DC).* 242:99–102.

Maylie, J., M. Irving, N. L. Sizto, and W. K. Chandler. 1987*a*. Comparison of Arsenazo III optical signals in intact and cut frog twitch fibers. *J. Gen. Physiol.* 89:41–81.

Maylie, J., M. Irving, N. L. Sizto, and W. K. Chandler. 1987*b*. Calcium signals recorded from cut frog twitch fibers containing antipyrylazo III. *J. Gen. Physiol.* 89:83–143.

Meissner, G., E. Darling, and J. Evelath. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Melzer, W., E. Rios, and M. F. Schneider. 1984. Time course of calcium release and removal in skeletal muscle fibers. *Biophys. J.* 45:637–641.

Ogawa, Y., and M. Tanokura. 1986. Steady-state properties of calcium binding to parvalbumins from bullfrog skeletal muscle: effects of Mg^{2+}, pH, ionic strength, and temperature. *J. Biochem.* 99:73–80.

Pape, P. C., M. Konishi, S. Hollingworth, and S. M. Baylor. 1990. Perturbation of sarcoplasmic reticulum calcium release and phenol red absorbance transients by large concentrations of fura-2 injected into frog skeletal muscle fibers. *J. Gen. Physiol.* 96:493–516.

Rios, E., and G. Pizarro. 1988. Voltage sensors and calcium channels of excitation-contraction coupling. *News Physiol. Sci.* 3:223–228.

Schneider, M. F., and B. J. Simon. 1988. Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *J. Physiol. (Lond.).* 405:727–745.

Schneider, M. F., B. J. Simon, and G. Szucs. 1987. Depletion of cal-

cium from the sarcoplasmic reticulum during calcium release in frog skeletal muscle. *J. Physiol. (Lond.)*. 392:167–192.

Simon, B. J., M. G. Klein, and M. F. Schneider. 1991. Calcium dependence of inactivation of calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. *J. Gen. Physiol.* 97:437–471.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Ca^{2+}. *J. Gen. Physiol.* 88:573–588.

Stephenson, E. 1978. Properties of chloride-stimulated ^{45}Ca flux in skinned muscle fibers. *J. Gen. Physiol.* 71:411–430.

Uto, A., H. Arai, and Y. Ogawa. 1991. Reassessment of fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium.* 12:29–37.