Effect of Fura-2 on Action Potential–stimulated Calcium Release in Cut Twitch Fibers from Frog Muscle

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ABSTRACT Cut fibers (striation spacing, 3.6–4.2 μm) were mounted in a double Vaseline-gap chamber and studied at 14–15°C. One or both of the Ca indicators fura-2 and purpurate-3,3’diacetic acid (PDAA) were introduced into the optical recording site by diffusion from the end pools. Sarcoplasmic reticulum (SR) Ca release was elicited by action potential stimulation. With resting [fura-2] = 0 mM at the optical site, the [Ca] transient measured with PDAA was used to estimate SR Ca release (Baylor, S.M., W.K. Chandler, and M.W. Marshall. 1983. Journal of Physiology. 344:625–666). With resting [fura-2] > 0 mM, the contribution from Ca complexation by fura-2 was added to the estimate. When resting [fura-2] was increased from 0 to 0.5–2 mM, both the amount of SR Ca release and the maximal rate of release were increased by ~ 20%. These results are qualitatively similar to those obtained in intact fibers (Baylor, S.M., and S. Hollingworth. 1988. Journal of Physiology. 403:151–192; Hollingworth, S., A. B. Harkins, N. Kurebayashi, M. Konishi, and S. M. Baylor. 1992. Biophysical Journal. 63:224–234) and are consistent with a reduction of Ca inactivation of SR Ca release produced by 0.5–2 mM fura-2. With resting [fura-2] ≥ 2 mM, the PDAA [Ca] transient was reduced to nearly zero and SR Ca release could be estimated from Δ[Cafura-2] alone. When resting [fura-2] was increased from 2–4 to 5–6 mM, both the amount of SR Ca release and the maximal rate of release were decreased by approximately half, consistent with a possible reduction of Ca-induced Ca release (Jacquemond, V., L. Csernoch, M. G. Klein, and M. F. Schneider. 1991. Biophysical Journal. 60:867–873) or a possible pharmacological effect of fura-2.

INTRODUCTION

In a vertebrate skeletal muscle fiber, normal activation of contraction begins with depolarization of the external membranes (including those of the transverse tubular system) and ends with the movement of Ca from the sarcoplasmic reticulum (SR) into the myoplasm where it can bind to the Ca-regulatory sites on troponin. SR Ca release

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appears to be controlled by several factors. One of these is the voltage across the
 tubular membranes. Another is the value of free [Ca], either in the bulk myoplasmic
 solution or near the myoplasmic surface of the SR release sites. Two suggested
 actions of Ca on SR Ca release are Ca-induced Ca release and Ca-dependent
 inactivation of SR Ca release (which will be called Ca inactivation of Ca release).

Ca-induced Ca release was discovered independently by Endo, Tanaka, and Ebashi
(1968) and Ford and Podolsky (1968). Both laboratories used frog skinned muscle
fibers for their experiments. Under suitable conditions, the addition of Ca to the
solution bathing the fiber was able to either initiate or facilitate SR Ca release, as
assessed by the development of tension. Subsequent experiments showed that the
level of myoplasmic free [Ca] that produced Ca-induced Ca release depended on the
extent of SR Ca loading and the value of free [Mg] in the bathing solution. With
normal values of these parameters, Ca-induced Ca release required that bulk
myoplasmic free [Ca] be elevated to a value \( \geq 100 \mu M \) (Endo, 1977). Since such a
large value is almost certainly never reached during normal action potential activa-
tion, at least in the bulk myoplasmic solution, serious doubts have been raised about
whether Ca-induced Ca release plays a significant role in the physiological regulation
of SR Ca release in vertebrate skeletal muscle (Endo, 1977). The mechanism may,
however, be important in the activation of cardiac muscle (Fabiato, 1985).

Ca inactivation of Ca release, on the other hand, appears to develop at the levels of
myoplasmic free [Ca] that accompany action potential stimulation. After a train of
action potentials, the peak rate of SR Ca release associated with the second and
subsequent action potentials is 0.1–0.2 times that associated with the first action
potential (see, for example, Fig. 8 B). Similarly, if a voltage-clamp depolarization is
given, the rate of SR Ca release usually increases initially, reaches an early peak, and
then decreases to an approximately steady level that may be as little as 0.1–0.2 times
the peak value. These decreases in the rate of SR Ca release, which, in voltage-clamp
experiments, are observed with even small levels of Ca release, were first described by
Baylor, Chandler, and Marshall (1983) and Melzer, Rios, and Schneider (1984).
Without direct evidence, Baylor et al. (1983) suggested that they were due to Ca
inactivation of Ca release produced by the elevation in free [Ca] that accompanied
the increase in SR Ca permeability. Subsequently, Schneider and collaborators
(Simon, Schneider, and Szucs, 1985; Schneider and Simon, 1988; Simon, Klein, and
Schneider, 1991) studied the decrease in SR Ca release in voltage-clamped cut fibers
under a variety of conditions and provided good evidence that Ca inactivation of Ca
release was indeed involved.

In recent years, there has been renewed interest in the possibility that Ca-induced
Ca release might play some role in normal activation in vertebrate skeletal muscle.
Fabiato (1984) studied rapid changes of [Ca] in microdissected fragments of fibers,
3–6 \( \mu m \) in diameter, and showed that SR Ca release could be produced by a small
increase in free [Ca] if it occurred rapidly. A 0.5-ms increase from 0.1 to 0.2 \( \mu M \)
produced a release in most experiments and a 5-ms increase from 0.1 to 0.6 \( \mu M \)
always worked. On the other hand, SR Ca release was not observed when free [Ca]
was increased gradually from 0.1 to 0.6 \( \mu M \) during a 100-ms period. This result
suggests that Ca-induced Ca release can be inactivated by Ca inactivation of Ca
release and that slow bath application of Ca to a skinned fiber might inactivate the
release mechanism without producing Ca-induced Ca release. In light of these considerations, it seemed important to reconsider the possibility that Ca-induced Ca release plays a role in the normal activation of skeletal muscle.

One way to evaluate the importance of Ca-induced Ca release or Ca inactivation of Ca release is to try to decrease its effect by reducing changes in myoplasmic free [Ca] with a rapidly reacting high affinity Ca buffer, such as BAPTA (Tsien, 1980) or fura-2 (Grynkiewicz, Poenie, and Tsien, 1985). A decrease in SR Ca release would be consistent with a reduction of Ca-induced Ca release, whereas an increase would be consistent with a reduction of Ca inactivation of Ca release. Studies along this line have been carried out in two laboratories and different results were obtained. Baylor and Hollingworth (1988) and Hollingworth, Harkins, Kurebayashi, Konishi, and Baylor (1992) injected fura-2 into intact fibers from *Rana temporaria* and studied SR Ca release with action potential stimulation. They found that fura-2, at concentrations as large as 3–4 mM, increased both the amount of Ca released from the SR and the peak rate of release. They attributed these increases to a reduction of Ca inactivation of Ca release produced by the Ca-binding capacity of fura-2. On the other hand, Jacquemond, Csernoch, Klein, and Schneider (1991) injected fura-2 or a mixture of fura-2 and BAPTA into cut fibers from *Rana pipiens* and found that SR Ca release was reduced: 2–3 mM fura-2 or 3–5 mM BAPTA completely eliminated the early peak, but not the smaller steady level, of SR Ca release elicited with voltage-clamp depolarization. They attributed this removal of the transient component of SR Ca release to a block of Ca-induced Ca release.

The experiments described in this and the following article (Jong, Pape, Chandler, and Baylor, 1993) were carried out to try to resolve the source of these apparent differences in the effects of fura-2. Since one set of the previous results was obtained with action potential-stimulated intact fibers from *Rana temporaria* and the other with voltage-clamped cut fibers from *Rana pipiens*, it seemed desirable to use action potential and voltage-clamp stimulation on the same type of muscle preparation. This was done with cut muscle fibers isolated from *Rana temporaria*, the frog that is usually used in our laboratories. In addition, to check for any species differences, three voltage-clamp experiments were carried out on fibers from *Rana pipiens*. This article describes our results with action potential stimulation and the following article (Jong et al., 1993) describes the results with voltage-clamp depolarization. Some of the results have been presented to the Biophysical Society (Pape, Jong, Chandler, and Baylor, 1993).

**METHODS**

The experiments with action potential stimulation were carried out at Yale University School of Medicine in March and June, 1992. Semitendinosus muscles were removed from *Rana temporaria* (obtained from Charles D. Sullivan, Inc., Nashville, TN and cold-adapted at 5°C) and then stretched and pinned in a dissection dish containing Ringer's solution. After partial dissection, the bathing solution was changed to a Ca-free, high K relaxing solution (Table 1A in Irving, Maylie, Sizto, and Chandler, 1987), which caused a contraction followed by relaxation. A short segment of one of the fibers was isolated (Hille and Campbell, 1976) and mounted in a double Vaseline-gap chamber (Kovacs, Rios, and Schneider, 1983). The end pool solution was changed to the internal solution (see below) and the end pool segments of the fiber were
permeabilized by a 2-min exposure to a 0.01% saponin solution followed by a thorough rinse. Ringer's solution was then introduced into the central pool and the fiber became partially repolarized (Fig. 5 in Irving et al., 1987). The chamber was then mounted on the experimental apparatus and the fiber was polarized to a holding potential of \(-90\) mV. The time that the fiber was depolarized in the relaxing solution varied but was usually about one-half hour. The time of partial polarization, before the holding potential was set to \(-90\) mV, was 10-15 min.

The Ringer's solution contained 120 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl\(_2\), 2.15 mM Na\(_2\)HPO\(_4\), and 0.85 mM NaH\(_2\)PO\(_4\) (pH 7.1). The internal solution used in the end pools contained 76 mM K-glutamate, 5.5 mM Na\(_2\)-ATP, 20 mM K\(_2\)-creatine phosphate, 6.8 mM MgSO\(_4\), 0.1 mM ethyleneglycol-bis-(\(\beta\)-aminoethyl ether)-N\(_2\),N\(_4\)-tetraacetic acid (EGTA), 5 mM K-phosphoenolpyruvate, and 5 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.0). The calculated level of free [Mg] was 1 mM.

Two different Ca indicators were used in the experiments: Kspurpurate-3,3'diacetate (PDAA; Hirota, Chandler, Southwick, and Waggner, 1989) and K\(_s\)fura-2 (Grynkiewicz et al., 1985). These were added to the end pool solutions, which were sometimes diluted slightly to prevent an increase in tonicity. PDAA was provided by Dr. Philip L. Southwick (Carnegie Mellon University, Pittsburgh, PA). Its concentration in the end pool solutions was determined from the value of the absorbance of the solution at 510 nm, measured in a spectrophotometer (model Lambda 3B; Perkin-Elmer Corp., Norwalk, CT; nominal bandpass, 1 nm). A value of 1.42 \times 10^4 \text{M}^{-1}\text{cm}^{-1} was used for \(\epsilon(510)\), the molar extinction coefficient of PDAA at 510 nm.

Ca-free K\(_s\)fura-2 was purchased from Molecular Probes, Inc. (Eugene, OR). The total concentration of fura-2, denoted by \([\text{fura-2}T]\), was estimated from the nominal amount of fura-2 provided by Molecular Probes, Inc.; it is equal to the sum of \([\text{fura-2}]\) and \([\text{Ca-fura-2}]\), the concentrations of Ca-free and Ca-bound fura-2, respectively. Spot checks with spectrophotometer measurements indicated that the estimate of \([\text{fura-2}T]\) was accurate within 10%. In many experiments, Ca was added to the solution to complex some of the indicator. The value of \([\text{Ca-fura-2}]\) was assumed to be equal to the concentration of added Ca.

The multi-wavelength apparatus that was used for the optical measurements as well as the methods of analysis are fully described in Irving et al. (1987), Maylie, Irving, Sizto, and Chandler (1987b, c), and Hirota et al. (1989). The experiments were carried out at 14°C except for fiber 619921, which was studied at 15°C. The striation spacing of the fibers was 3.6-4.2 \(\mu\)m. The statistical significance of a difference between two sets of results was determined with the two-tailed t test; if \(P \leq 0.05\), the difference was considered to be significant.

**Measurement and Analysis of Resting Absorbance Signals**

Our optical apparatus can be used to measure simultaneously the intensities of transmitted light at three different wavelengths and two planes of linear polarization. Before an indicator was introduced into the end pools, the six intensities were measured both with the fiber positioned in the light path and with the fiber removed. At each of the three wavelengths, the intrinsic absorbance of the fiber (defined here to include reductions in light intensity due to both absorbance and light scattering) for 0° and 90° linearly polarized light was calculated from the logarithm (base 10) of the ratio of incident to transmitted light (Eq. 1 in Irving et al., 1987). The intrinsic absorbance of the fiber at that wavelength was taken as the 1:2 average of the values for 0° and 90° light (see description of mode 1 operation on pages 7-9 of Irving et al., 1987).

After the intrinsic absorbance of the fiber had been measured, a solution containing either fura-2 or PDAA was introduced into the end pools so that the indicator could diffuse into the optical recording site in the middle of the fiber in the central pool region. The myoplasmic concentration of indicator at the optical site was estimated from the value of indicator-related absorbance. This was calculated from the absorbance of the fiber containing indicator minus
the intrinsic absorbance determined before indicator was added to the end pools. Allowance
was made for changes in both the intrinsic absorbance of the fiber and the intensity of the light
source, with the procedure described on pages 44-45 of Maylie et al. (1987b). Throughout this
paper, the absorbance of a fiber for light of wavelength \( \lambda \) is denoted by \( A(\lambda) \).

When fura-2 was used alone, the resting concentration of fura-2 was estimated from
measurements of indicator-related \( A(410) \) or \( A(420) \), since the absorbance of Ca-fura-2 at these
wavelengths is sufficiently small that it may be taken as zero. The values of the apparent molar
extinction coefficient for the wavelengths passed by our filters were determined from measure-
ments carried out with calibration solutions inside a 1-mm cuvette mounted on our optical
apparatus; the resting concentration of fura-2 in these solutions was estimated from the value of
absorbance at 420 nm, determined with a spectrophotometer (model Lambda 3B; Perkin-
Elmer Corp.; nominal bandpass, 1 nm) and the assumption that \( e(420) = 0.32 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \)
(Baylor and Hollingworth, 1988).

When PDAA was used alone, its resting myoplasmic concentration was determined from
measurements of indicator-related \( A(510) \). When PDAA and fura-2 were used together, PDAA
was always applied first. When its concentration at the optical site reached 2-3 mM, the ratio of
\( A(410)/A(510) \) or \( A(420)/A(510) \) was determined and then fura-2 was introduced into the end
pools. Subsequent estimates of the resting myoplasmic concentration of PDAA were obtained
directly from indicator-related \( A(510) \), since neither fura-2 nor Ca-fura-2 absorbs 510-nm light.
The resting myoplasmic concentration of fura-2 was estimated from the value of indicator-
related \( A(410) \) or \( A(420) \) after correction for the contribution from PDAA. This contribution
was assumed to equal the product of PDAA-related \( A(510) \) times the value of PDAA-related
\( A(410)/A(510) \) or \( A(420)/A(510) \) that was measured before fura-2 had been introduced into the
end pools.

**Measurement and Analysis of Resting Fluorescence Signals**

The fluorescence from Ca-free fura-2 was also determined in our experiments. For this
purpose, a 420-nm (30-nm bandpass) filter was placed in the incident light path and a filter
that passed 480-600 nm light was placed in the path of the transmitted light. The fluorescence
intensities are expressed in terms of the voltage developed across the 10-M\( \Omega \) feedback
resistance used in the photodiode circuit.

The intensity of the measured fluorescent light is influenced by several factors (see, for
example, Baylor, Chandler, and Marshall, 1981). These include the intensity of the light
incident on the fiber, the concentration of indicator, the indicator's quantum efficiency for
fluorescence, the extent to which the incident and fluorescent light are absorbed or scattered by
the fiber, the numerical aperture used to collect the emitted light, and the efficiency of the
photodetector. Let \( I \) represent the intensity of the light incident on the fiber and \( A_I \) (1 for
incident), the absorbance of the fiber (both intrinsic and indicator related) for light of this
wavelength. At a distance \( x \) along the light path from the illuminated surface, the intensity of
light \( I_x \) is given by

\[
I_x = I \cdot 10^{-A \pi x/d}
\]

and its derivative is given by

\[
dI_x = -(\ln 10) \cdot (A_I/d) \cdot I_x \, dx
\]

where \( d \) denotes the diameter of the fiber.

Some of the light that is absorbed by the fiber will be absorbed by fura-2 and, depending on
the indicator's quantum efficiency for fluorescence, will be emitted as fluorescent light. Let \( dF \),
represent the fluorescent light that is emitted between \( x \) and \( x + dx \) and then propagates along
the light path toward the photodiode:

\[ dF_x = -f'k dI_x \]  

(3)

\( f \) represents the fraction of the light absorbed or scattered by the fiber that is absorbed by fura-2; thus, \( f'A \) represents the fura-2-related absorbance, \( A_{\text{ind}} \). \( k \) is a factor that is equal to the fluorescence efficiency of the indicator times the fraction of fluorescent light that would be collected by the optical apparatus and eventually be measured by the photodiode if none of it were absorbed or scattered by the fiber.

Since some of the fluorescent light \( dF_x \) may be absorbed or scattered by the fiber, the total intensity of fluorescent light \( F \) that is measured by the photodiode will be given by

\[ F = \int_0^d 10^{-A_{\text{fib}} x/d} dF_x \]  

(4)

\( A_{\text{f}} \) (\( F \) for fluorescence) represents the absorbance of the fiber for light of the wavelength of the fluorescent light.

Eqs. 1–4 can be combined to give the following relation between the ratio of the intensities of measured fluorescent light and incident light:

\[ \frac{F}{I} = \frac{\ln 10^{-A_{\text{f}} x/d} - A_{\text{fib}}}{A_1 - A_F} A_{\text{ind}} k \]  

(5)

This equation is similar to Eq. 5 in Baylor et al. (1981).

The terms \( A_1 \) and \( A_F \) contain, in principle, contributions from the absorbance of indicators (fura-2 and, if present, PDAA) and from the intrinsic absorbance of the fiber. If the values of \( A_1 \) and \( A_F \) are sufficiently small, Eq. 5 can be replaced by the limiting relation,

\[ \frac{F}{I} = (\ln 10) A_{\text{ind}} k \]  

(6)

in which case the value of \( F/I \) is directly proportional to the value of fura-2-related absorbance.

With either Eq. 5 or 6, it is important to note that the empirically determined value of \( k \) represents the actual value of \( k \) times the ratio of the efficiencies of the detector at the emission and excitation wavelengths.

Measurement and Analysis of Signals during Fiber Activity

The electrical and optical signals were amplified and filtered by eight-pole Bessel filters before being sampled by an A/D converter connected to a multiplexer. The Bessel filter frequency was 2 kHz.

The optical signals were recorded simultaneously at three different wavelengths. The longest wavelength was sufficiently long that the indicator(s) did not absorb light. The signal at this wavelength gave the waveform of the intrinsic absorbance change of the fiber. It was scaled to give the estimated intrinsic change at the two shorter wavelengths and then subtracted from the corresponding raw absorbance signals to give the indicator-related signals. The procedure used to determine the scaling constants for the intrinsic signal is described on pages 44–45 and in Fig. 4 in Maylie et al. (1987b). It relies on the assumption that the intrinsic signal at the two shorter wavelengths has the same waveform as the long wavelength signal but an amplitude that varies according to wavelength raised to some power. It also relies on the assumption that the Ca signal at the two shorter wavelengths has the same waveform, which, in general, is not the case if the signal contains contributions from both PDAA and fura-2. In experiments with both PDAA and fura-2, PDAA was always used first and the scaling constants were determined with PDAA before fura-2 was introduced into the end pools.
Changes in myoplasmic free [Ca], Δ[Ca], were estimated with PDAA's ΔA(570)/A(510) signal, as described in Hirota et al. (1989). Changes in myoplasmic [fura-2], Δ[fura-2], were estimated from the indicator's ΔA(410) or ΔA(420) signal, both of which contain negligible contributions from the absorbance of Ca-fura-2. Since Δ[fura-2] was recorded during a period that lasted no more than 1–2 s, [fura-2] was essentially constant during a measurement; hence, Δ[Ca-fura-2] = −Δ[fura-2]. In experiments with both PDAA and fura-2 at the optical site, the contribution of changes in PDAA absorbance to the ΔA(410) or ΔA(420) signal was first estimated and subtracted from the indicator-related ΔA(570) or ΔA(420) signal to give the fura-2-related ΔA(410) or ΔA(420) signal. The PDAA contribution to ΔA(410) or ΔA(420) was assumed to equal the indicator-related ΔA(570) signal (which does not contain any contributions from fura-2) scaled by the value of indicator-related ΔA(410)/ΔA(570) or ΔA(420)/ΔA(570) that was measured with PDAA before fura-2 was introduced into the end pools.

Except for Fig. 2, the signal-to-noise of the optical signals was improved by use of a digital Gaussian filter (Colquhoun and Sigworth, 1983). The intrinsic signal recorded at the longest wavelength was filtered by a 0.05-kHz Gaussian filter and the indicator-related optical signals were filtered by a 0.5-kHz Gaussian filter. This filtering had little effect on the signals reported in this paper. The peak value of dA[CaT]/dt was estimated to be reduced by a few percent in some experiments, but by no more than 5%.

A period of at least 3 min was allowed for recovery after a single action potential before a subsequent stimulation was done. A 5-min period was allowed after a train of action potentials.

Estimate of the Concentration of Ca-free Fura-2 from Resting Fluorescence and Active Changes in Fluorescence and Absorbance

The estimates of the resting concentration of fura-2 that were obtained from the resting values of fura-2-related A(410) or A(420) were compared with those obtained with a second method, which used the resting value of F and the values of ΔF and ΔA(410) or ΔA(420) that accompanied stimulation. If the values of A1 and AF are sufficiently small that Eq. 6 can be used, ΔF/I is given by

\[
\frac{\Delta F}{I} = (\ln 10) \Delta A_{\text{Ind}} \cdot k
\]

Eq. 7 follows from Eq. 6 since I and k should not be affected by stimulation. Eqs. 6 and 7 can be combined to give

\[
A_{\text{Ind}} = \Delta A_{\text{Ind}} \cdot (F/\Delta F)
\]

Since A_{\text{Ind}} is directly proportional to the resting concentration of fura-2, Eq. 8 is essentially the same as Eq. 6 in Baylor and Hollingsworth (1988).

With large concentrations of fura-2, however, such as those used in the experiments in this article, Eq. 5 rather than Eq. 6 should be used to relate measurements of fluorescence to values of absorbance. Thus, Eq. 5 establishes the relations between resting A_{\text{Ind}} and F and between A_{\text{Ind}} + ΔA_{\text{Ind}} and F + ΔF, and an iterative computer program can be used to calculate A_{\text{Ind}} from the values of ΔF/F and ΔA_{\text{Ind}}. An exact calculation of this sort requires knowledge of the values of the resting intrinsic absorbance of the fiber (and of PDAA, if present) at the wavelengths of the incident and fluorescent light as well as values of their changes. It turns out, however, that these values have little influence on the estimate of A_{\text{Ind}}. For example, in some of the experiments with PDAA, a substantial fraction of the fluorescent light from fura-2 was absorbed by PDAA. This reduced both the F and ΔF signals by an amount that is expected to be the same for both signals. Since the estimate of A_{\text{Ind}} depends on the ratio of F and ΔF and not on their individual values, it is not expected to be influenced by the presence of PDAA.
If the effects of the intrinsic signals and any PDAA signals are ignored, the estimate of $A_{\text{ind}}$ is changed by < 1% and its value is given by

$$A_{\text{ind}} = \log_{10} \left[ 1 + (1 - 10^{-A_{\text{ind}}}) \frac{F}{\Delta F} \right]$$

(9)

Eq. 9 was used to calculate the values of $A_{\text{ind}}$ that were used to give the values of the resting concentration of fura-2 indicated by x's in Figs. 1A, 5B, 7D, and 9.

**Table 1**

| Parameters Associated with the Estimation of SR Ca Release from Myoplasmic Free [Ca] |
|---------------------------------|------------------|------------------|------------------|------------------|
| Protein buffer                  | Concentration of sites | Cation | $k_1$ | $k_{-1}$ |
| A Model 1 (Baylor et al., 1983)  |                      |       |       |          |
| Troponin                        | 240                | Ca     | $0.575 \times 10^6$ | 115    | 2.0    |
| Parvalbumin                     | 1,500              | Ca     | $1.25 \times 10^6$ | 0.5    | 0.004  |
|                                 |                    | Mg     | $3.30 \times 10^4$ | 3.0    | 90.9   |
| B Model 2 (Jacquemond et al., 1991) |        |       |       |          |
| Troponin                        | 250                | Ca     | $1.3 \times 10^6$  | 1,000  | 7.7    |
| Parvalbumin                     | 1,000              | Ca     | $1.6 \times 10^6$  | 1.5    | 0.0094 |
|                                 |                    | Mg     | $4.0 \times 10^4$  | 5.5    | 137.5  |
| SR Ca pump                      | 200                | Ca     | (instantaneous)    | 1.0    |        |

Column 1 gives the name of the intrinsic Ca buffer and column 2 gives its myoplasmic concentration. The term troponin refers to its Ca-regulatory sites and the term parvalbumin refers to its Ca,Mg sites. Column 3 gives the cation, Ca or Mg, that the binding parameters in columns 4-6 describe. Columns 4 and 5 give, respectively, the forward ($k_1$) and backward ($k_{-1}$) rate constants for the reaction between the cation and the protein buffer. Column 6 gives the $K_D$, the dissociation constant of the reaction, which is equal to $k_{-1}/k_1$.

Model 1 (part A) is the same as model 2 used by Baylor et al. (1983) except that the concentration of Ca,Mg sites on parvalbumin is 1.5 mM, taken from Hou, Johnson, and Rall (1991), instead of 1 mM. Model 2 (part B) is the same as that used by Jacquemond et al. (1991) and is taken from Melzer, Rios, and Schneider (1984, 1987) and Klein, Simon, and Schneider (1990); the rate of Ca pumping, which is added to $d\Delta [Ca]/dt$, is assumed to be equal to $V_{\text{max}}$ times the fractional occupancy of the sites, with $V_{\text{max}} = 0.9 \mu M-ms$. The calculations with model 2 were done with the unscaled myoplasmic $\Delta [Ca]$ waveform and with the waveform scaled by 0.25, denoted by models 2:Δ[Ca] and 2:0.25Δ[Ca], respectively.

**Estimates of SR Ca Release from the Myoplasmic Free [Ca] Transient**

Two methods were used to estimate SR Ca release from the myoplasmic [Ca] signal measured with PDAA. The basic idea in each method is to calculate the myoplasmic concentrations of Ca that are bound to the Ca-regulatory sites on troponin (denoted by [CaTrop]), the Ca,Mg sites on parvalbumin ([CaParv]), and, in the model used by Jacquemond et al. (1991), the SR Ca pump ([CaPump]). The concentrations and rate constants of the various Ca buffers used in the models are given in Table I. $\Delta [Ca_T]$, the change in the total concentration of Ca in the myoplasmic solution after stimulation, is estimated from $\Delta [Ca_T] = \Delta [Ca] + \Delta [Ca_{PDAA}] + \Delta [Ca_{Trop}] + \Delta [Ca_{Parv}] + \Delta [Ca_{Pump}] + \Delta [Ca_{fura-2}]$; if fura-2 is absent, $\Delta [Ca_{fura-2}] = 0$. The time derivative of $\Delta [Ca_T]$, $d\Delta [Ca_T]/dt$, is taken to represent the rate of Ca release from the SR resulting from stimulation of the fiber.
In the model used by Jacquemond et al. (1991) (model 2), the change in the rate of translocation of Ca by the Ca pump is also added to $d[\Delta(Ca)]/dt$; the rate of translocation is assumed to be equal to $V_{max}$ times the fractional occupancy of the pump sites, with $V_{max} = 0.9$ $\mu$M/ms. Calculations with this model were carried out with the unscaled $\Delta[Ca]$ signal (denoted by $\Delta[Ca]$). Since Jacquemond et al. (1991) used antipyrulazo III to estimate $\Delta[Ca]$ signals, and since the amplitude of the $\Delta[Ca]$ signal measured with antipyrulazo III is expected to be only 0.2–0.25 times that estimated with PDAA (Maylie, Irving, Sizto, Boyarski, and Chandler, 1987a; Maylie et al., 1987c; Hirota et al., 1989; Konishi and Baylor, 1991; Konishi, Hollingworth, Harkins, and Baylor, 1991), model 2 calculations were also carried out with the $\Delta[Ca]$ signal scaled by 0.25 so that our results could be compared with those of Jacquemond et al. (1991). Throughout the text, model 2:$\Delta[Ca]$ will be used to denote model 2 with the unscaled $\Delta[Ca]$ signal; similarly, model 2:0.25$\Delta[Ca]$ will be used to denote model 2 with the $\Delta[Ca]$ signal scaled by 0.25.

In all calculations, resting myoplasmic free [Ca] and [Mg] were assumed to be 50 nM and 1 mM, respectively.

**RESULTS**

Previous studies at 16°C on intact fibers stimulated by a single action potential showed that, when the concentration of myoplasmic fura-2 was increased from 0 to 1 mM (Baylor and Hollingworth, 1988) or from 0 to 3 mM (Hollingworth et al., 1992), the amount of Ca released from the SR, denoted by $\Delta[Ca_T]$, increased from a value of ~300 $\mu$M to 500–600 $\mu$M. In the same studies, the peak rate of Ca release, denoted by $d\Delta[Ca_T]/dt$, increased from ~100 $\mu$M/ms to values as large as 200 $\mu$M/ms. As mentioned in the Introduction, these authors attributed this potentiation of SR Ca release to the ability of fura-2, by complexing Ca and thereby reducing $\Delta[Ca]$, to reduce Ca inactivation of Ca release.

The experiments reported in this article were carried out on action potential-stimulated cut muscle fibers. The main aim was to find out whether 1–3 mM fura-2 potentiates SR Ca release, similar to the potentiation just described for intact fibers, or suppresses it, as reported by Jacquemond et al. (1991) in voltage-clamped cut fibers. We will first describe experiments carried out with fura-2 alone and then describe experiments with fura-2 plus PDAA.

**Diffusion of Fura-2 into Cut Fibers from the End Pool Solutions**

In the experiments of Baylor and Hollingworth (1988), Jacquemond et al. (1991), and Hollingworth et al. (1992), fura-2 was introduced into fibers by pressure injection, whereas in our experiments it was introduced by diffusion from the end pools. One advantage of the diffusion method is that it avoids possible damage from microinjection. Another advantage is that rather large concentrations of fura-2, up to 8 mM, can be introduced and maintained for tens of minutes at the optical recording site. A disadvantage of the method is that it takes some tens of minutes for indicator to diffuse from the end pools to the optical site, which is located in the middle of the fiber in the central pool region.

In the experiment illustrated in Figs. 1–4, the end pool solution contained a total concentration of fura-2, denoted by $[fura-2_T]$, of 4 mM; 1 mM Ca was added to the
solution so that \([Ca_{	ext{free}}-2] = 1 \text{ mM}\) and \([\text{fura}-2\text{-}] = 3 \text{ mM}\). Thus, the fraction of fura-2 that was complexed with Ca, 0.25, was close to 0.3, the fraction used in the solutions injected by Jacquemond et al. (1991) and Hollingworth et al. (1992). The filled circles in Fig. 1A show the values of the resting myoplasmic concentration of fura-2 at the optical site, estimated from the values of indicator-related absorbance at 420 nm as described in Methods (Ca-fura-2 does not absorb 420-nm light). They are plotted as a function of time after the introduction of indicator into the end pools.

![Graph A](image)

**Figure 1.** The concentration of resting fura-2 at the optical recording site plotted as a function of time after the addition of 4 mM fura-2 with 1 mM Ca to the end pool solutions. (A) The filled circles show (Ca-free) [fura-2] estimated from indicator-related A (420), as described in Methods. The four x's show resting [fura-2] estimated from measurements of \(\Delta A(420)\) and \(\Delta F/F\), as described in Methods (see Eq. 9). The curve shows a least-squares fit of Eqs. 6 and 8 in Maylie et al. (1987b), which were derived from diffusion plus linear reversible binding; \(D = 0.52 \times 10^{-6} \text{ cm}^2/\text{s}\) and \((R + 1) = 1.31\). The two vertical ticks indicate when the records in Figs. 2 and 3 were taken. (B) The filled circles show resting fluorescence from the same experiment. The vertical tick indicates when the \(\Delta F\) trace in Fig. 2 was taken. The theoretical curve was calculated from the concentration curve in A and Eq. 5 in Methods; see text for additional information. Fiber reference, 318921; sarcomere spacing, 3.6 \(\mu\text{m}\). Range of values, beginning to end of the experiment: fiber diameter, 108–106 \(\mu\text{m}\); holding current, \(-26\) to \(-38\) nA; action potential amplitude, 132–126 mV. Fura-2 was introduced into the end pools 30 min after saponin treatment of the end pool segments.

The theoretical curve in Fig. 1A provides a good description of the time course of the resting concentration of fura-2 at the optical site. It represents a least-squares fit of the data by the solution of the usual one-dimensional diffusion equation with the assumption that any myoplasmic binding of indicator is linear, rapid, and reversible (Eqs. 6 and 8 in Maylie et al., 1987b). Since only Ca-free fura-2 is detected optically, this analysis relies on the implicit assumption that there is no gradient of free [Ca] along the muscle fiber, an assumption that may not be strictly correct. According to
the fit, $D/(R + 1) = 0.40 \times 10^{-6}$ cm$^2$/s and $(R + 1) = 1.31$; thus, $D = 0.52 \times 10^{-6}$ cm$^2$/s. $D/(R + 1)$ represents the apparent diffusion constant of fura-2, $R$ represents the ratio of bound indicator to free indicator, and $D$ represents the actual diffusion constant of fura-2 in the myoplasm; bound indicator itself is assumed to be immobile.

Similar indicator diffusion curves were obtained in 10 fibers from $Rana temporaria$ with $[fura-2_T] = 4-8$ mM in the end pools. Three fibers, including the one in Fig. 1, were studied with action potential solutions and fura-2 as the only indicator. The average values in these three experiments were $D/(R + 1) = 0.42 \times 10^{-6}$ cm$^2$/s (SEM, $0.05 \times 10^{-6}$ cm$^2$/s), $(R + 1) = 1.09$ (SEM, 0.11), and $D = 0.45 \times 10^{-6}$ cm$^2$/s (SEM, $0.05 \times 10^{-6}$ cm$^2$/s). The other seven fibers were studied with both PDAA and fura-2; three were studied with action potential solutions and four with voltage-clamp solutions (Jong et al., 1993). The average values from all 10 experiments were $D/(R + 1) = 0.43 \times 10^{-6}$ cm$^2$/s (SEM, $0.04 \times 10^{-6}$ cm$^2$/s), $(R + 1) = 1.27$ (SEM, 0.06), and $D = 0.54 \times 10^{-6}$ cm$^2$/s (SEM, $0.04 \times 10^{-6}$ cm$^2$/s). The values of $D/(R + 1)$, $(R + 1)$, and $D$ showed no obvious dependence on the concentration of fura-2, on the ratio $[Cafura-2]/[fura-2_T]$ in the end pool solutions, or on whether PDAA was used.

The mean value of $D/(R + 1)$, $0.43 \times 10^{-6}$ cm$^2$/s, is similar to the value $0.36 \times 10^{-6}$ cm$^2$/s obtained by Baylor and Hollingworth (1988) in intact fibers and the value $0.40 \times 10^{-6}$ cm$^2$/s obtained by Csernoch, Jacquemond, and Schneider (1993) in cut fibers. These values are slightly less than half the value $0.9-1.0 \times 10^{-6}$ cm$^2$/s estimated by Baylor and Hollingworth (1988) for an unbound molecule with the molecular weight of fura-2 (637), consistent with the idea that more than half the fura-2 inside a fiber is bound or sequestered to myoplasmic constituents. On the other hand, the average value of $(R + 1)$ was 1.27 instead of a value greater than 2. This suggests that, by this criterion, only a small fraction of fura-2 appears to be bound or sequestered, in agreement with the results of Klein, Simon, Szucs, and Schneider (1988). These authors suggested that fura-2 might be bound to myoplasmic constituents but excluded from the myofilament lattice so that the total concentration inside a fiber would be approximately equal to that in the end pools. Konishi, Olson, Hollingworth, and Baylor (1988) have provided additional evidence, unrelated to diffusion measurements, that a large fraction of fura-2 (0.7–0.8) is bound within the fiber.

The filled circles in Fig. 1B, taken from the same experiment as Fig. 1A, show the values of resting fluorescence, denoted by $F$, at the optical site plotted as a function of time after the introduction of fura-2 into the end pools. The intensity of the fluorescent light was low and consequently the data show some scatter. The theoretical curve for $F$ was calculated from Eq. 5 with the indicator-related $A(420)$ vs. time curve that was used to calculate the concentration vs. time curve in Fig. 1A. The value of $k$ times the ratio of the efficiencies with which the photodiode measures fluorescent and incident light was chosen to give a least-squares fit to the data, 0.094. A similar analysis was carried out in two other experiments in which 7 mM fura-2 + 1 mM Cafura-2 and 8 mM fura-2 alone were used in the end pool solutions. The fits were somewhat less good than the one in Fig. 1B: in both fibers, the curve lay slightly below the data at early times and a few percent above the data at late times. The
values of \( k \) times the photodiode efficiency ratio were 0.102 (fiber 615921, [fura-2] = 7 mM and [Cafura-2] = 1 mM) and 0.106 (fiber 619921, [fura-2] = 8 mM).

**Estimates of the Resting Concentration of Fura-2 from \( \Delta F/F \) and \( \Delta A(420) \)**

The resting concentration of fura-2 at the optical site can also be estimated from the value of \( F \) and the values of \( \Delta F \) and indicator-related \( \Delta A(420) \) that accompany stimulation, as described below. The four \( \times \)'s in Fig. 1A show values obtained with this method. They are in reasonable agreement with the filled circles (see also Figs. 5 B, 7 D, and 9), consistent with the idea that the estimate of indicator-related \( A(410) \) or \( A(420) \) in cut fibers is reliable and can be used to estimate the resting concentration of fura-2.

**Figure 2. Effects of action potential stimulation on optical signals from a fiber containing fura-2.** The top trace shows the action potentials recorded from the voltage-measuring end pool. The fiber was first stimulated to give a single action potential and, after 150 ms, was stimulated at 50 Hz to give a train of 50 action potentials. During the single action potential and the first nine action potentials of the train, each channel of data was stored at a rate of one point per 0.24 ms. Thereafter, the rate was one point per 1.2 ms. The second trace shows \( \Delta A(480) \). The third trace shows indicator-related \( \Delta A(420) \); this was obtained by subtraction of the \( \Delta A(480) \) signal, appropriately scaled (see Methods), from the uncorrected \( \Delta A(420) \) signal (not shown). In this and subsequent figures, the mean value of the prestimulus baseline was subtracted from each trace. The calibration bar marked -0.01 applies to both the \( \Delta A(480) \) and \( \Delta A(420) \) traces. The bottom trace shows \( \Delta F \) scaled so that the vertical excursion of the signal matches that of \( \Delta A(420) \). The \( \Delta A \) signals were taken at the time marked by the vertical tick above the filled circle in Fig. 1A. The \( \Delta F \) signal was taken at the time marked by the vertical tick below the \( \times \) in Fig. 1A and by the vertical tick in Fig. 1B. In this and subsequent figures, time = 0 corresponds to the time of stimulation of the first action potential. The horizontal bar in the lower right-hand part of the figure shows the time over which the \( \Delta A(420) \) and \( \Delta F \) signals were averaged for the determination of resting [fura-2] indicated by the right-most \( \times \) in Fig. 1A. For this determination, the value of indicator-related \( A(420) \) was estimated from Eq. 9 to be 0.0344, with \( \Delta A(420) = -0.02310 \), \( \Delta F = -143.5 \ \mu V \), and \( F = 216.8 \ \mu V \). Same experiment as Fig. 1; [fura-2] = 3.646–3.695 mM.

Fig. 2 shows the traces that were used to estimate the largest value of \( \times \) in Fig. 1A (marked by the vertical tick at 138.2 min). The top trace in Fig. 2 shows the action potentials. During this run, the fiber was stimulated to give one action potential and then, after a recovery period of 150 ms, 50 action potentials at 50 Hz. The sampling interval associated with the first 1,560 points was 0.24 ms. Thereafter, it was 1.2 ms
(see pages 6–7 in Irving et al., 1987), which was not sufficiently brief to reliably monitor the amplitude of each action potential. The second trace in Fig. 2 shows $\Delta A(480)$. Since neither fura-2 nor Cafura-2 absorbs 480-nm light, this trace gives the intrinsic signal at this wavelength. The third trace shows indicator-related $\Delta A(420)$. It was obtained by subtraction of the intrinsic $\Delta A(480)$ signal, suitably scaled, from the uncorrected $\Delta A(420)$ signal (not shown; see Methods).

The first three traces in Fig. 2 were obtained from the same run. Quasi-white light was used for the incident beam and the transmitted light was split into three beams, two of which were used for the $\Delta A(480)$ and $\Delta A(420)$ signals. 5 min earlier (indicated by the vertical tick in Fig. 1 B), $F$ and $\Delta F$ were measured with a 420-nm filter in the incident light path and a 480–600-nm filter in the transmitted light path. The $\Delta F$ signal, shown at the bottom of Fig. 2, is much noisier than the $\Delta A(420)$ signal for two reasons. The indicator-related absorbance was sufficiently large that, in the absence of dark current noise, the theoretical signal-to-noise ratio of the absorbance signal is expected to be larger than that of the fluorescence signal (Ross, Salzberg, Cohen, and Davila, 1974). In addition, the dark current noise of the photodiodes makes only a small contribution to the $\Delta A(420)$ signal but a substantial contribution to the $\Delta F$ signal, since the intensity of the fluorescent light was low.

For the estimate of the value of $\times$ at 138.2 min in Fig. 1 A, the values of $\Delta A(420)$ and $\Delta F$ in Fig. 2 were averaged during the interval indicated by the horizontal bar in the lower right-hand part of the figure. Since the $\Delta A(420)$ and $\Delta F$ traces were taken at different times separated by 5 min, the value of $\Delta A(420)$ was averaged from bracketting measurements, one taken 5 min before the $\Delta F$ trace and another taken 5 min after it. The mean value of $\Delta A(420)$, $-0.02310$, corresponds to a decrease in myoplasmic [fura-2], or an increase in [Cafura-2], of 2.072 µM.

**Ca Content of the SR**

$\Delta A(420)$ and $\Delta F$ signals usually reach plateau levels after a train of 50 action potentials, as was observed in the experiment in Fig. 2. The value of $\Delta[Cafura-2]$ during the plateau is influenced by SR Ca content and the value of the resting concentration of fura-2. If the resting concentration of fura-2 is less than the SR Ca content (expressed as myoplasmic concentration), the $\Delta[Cafura-2]$ signal would be expected to show a plateau when all the resting fura-2 becomes complexed with Ca. On the other hand, if the resting concentration of fura-2 is greater than the SR Ca content, the $\Delta[Cafura-2]$ signal would be expected to show a plateau when all the readily releasable Ca leaves the SR and becomes complexed with some, but not all, of the fura-2. In this case, the plateau level should be equal to the concentration of the readily releasable Ca inside the SR times the fractional amount of released Ca that is complexed by fura-2. If the resting concentration of fura-2 is much greater than the SR Ca content, it seems likely that this fractional amount would approach unity and the plateau concentration of Cafura-2 would approach the concentration of readily releasable Ca that was present inside the SR before stimulation.

At the time of the measurements in Fig. 2, the values of $\Delta[Cafura-2]$ and resting [fura-2] were 2.072 µM and 3.646–3.695 mM, respectively. Hence, the readily releasable SR Ca content is estimated to be $\approx 2.072$ µM. (Note that here and
elsewhere, the resting concentration of an indicator will be expressed as millimolar 
and changes during activity as micromolar.)

Changes in [Cafura-2] Produced by a Single Action Potential and by a Tetanus 
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Fig. 3 shows traces associated with the first action potential in Fig. 2. The first two 
traces show, respectively, the action potential and the A[Cafura-2] signal, estimated 
from the indicator-related ΔA(420) signal in Fig. 2. In the 2–6-ms period after the 
upstroke of the action potential, Δ[Cafura-2] increased 213 μM.

The complexation of Ca by fura-2 is expected to obey the mass action equation,
\[
d[Cafura-2]/dt = k_1[Ca][fura-2] - k_{-1}[Cafura-2]
\]
(10)
\[
d[Cafura-2]/dt = k_1[Ca][fura-2] - (k_1[Ca] + k_{-1})[Cafura-2]
\]
(11)

If [Cafura-2] is defined by [Cafura-2] = [Ca] + [Cafura-2], Eq. 10 can be written

\[
d[Cafura-2]/dt = k_1[Ca_T][fura-2] - (k_1[fura-2] + k_{-1})[Cafura-2]
\]
(12)

At this stage, it is useful to express each of the variables, [Cafura-2], [fura-2], [Ca_T], 
and [Ca], as a sum of the resting value (denoted by subscript 0) and the change with 
respect to this value (denoted by Δ). Thus, [Cafura-2] = [Cafura-2]_0 + Δ[Cafura-2], 
and so forth. If the resting concentrations are at equilibrium, Eq. 12 becomes

\[
dΔ[Cafura-2]/dt = k_1[fura-2]Δ[Ca_T]
\] 
- (k_1[fura-2] + k_1[Ca]_0 + k_{-1})Δ[Cafura-2] 
(13)

In many of our experiments, fura-2 was present in sufficient concentration to be 
able to complex almost all the Ca released from the SR. In this case, the amount 
of Ca released from the SR is approximately equal to Δ[Ca_T].
Eq. 11 states that $[Cafura-2]$ tracks free $[Ca]$ with an apparent rate constant equal to $k_t[Ca] + k_{-1}$, which, for small values of $[Ca]$, approaches a lower limit of $k_{-1}$. According to the temperature jump measurements of Kao and Tsien (1988), carried out with 5 μM fura-2 in a 140 mM KCl solution at 20°C, $k_1 = 6 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 97 \text{s}^{-1}$. Consequently, the dissociation constant of Ca and fura-2, given by $K_D = k_{-1}/k_1$, is 0.162 μM and the value of the apparent rate constant, $k_t[Ca] + k_{-1}$, is $\geq 97 \text{s}^{-1}$. The values of $k_1$, $k_{-1}$, and $K_D$ obtained by Kao and Tsien (1988) are given in the first row of columns 4–6 in Table II.

Similarly, Eq. 13 states that $\Delta[Cafura-2]$ tracks $\Delta[Ca]$ with an apparent rate constant given by $k_t[Ca] + k_t[Ca_0] + k_{-1}$, which is approximately equal to $k_t[fura-2] + k_{-1}$. With the rate constants given by Kao and Tsien (1988), even a small concentration of fura-2, 1 mM for example, gives a large value for this lower limit, $600,097 \text{s}^{-1}$. Thus, with the rate constants given by Kao and Tsien (1988), the Cafura-2 signal is expected to track free $[Ca]$ with a delay $\leq 10 \text{ms}$ and, if $[fura-2] \geq 1 \text{mM}$, to track $\Delta[Ca]$ with a delay $< 2 \mu\text{s}$.

### Table II

| (1) Reference | (2) Conditions          | (3) [fura-2] | (4) $k_1$ | (5) $k_{-1}$ | (6) $K_D$ |
|---------------|-------------------------|-------------|-----------|-------------|-----------|
| Kao and Tsien (1988) | 140 mM KCl, 20°C      | 0.005       | $6 \times 10^8$      | 97         | 0.162     |
| Klein et al. (1988)   | Cut muscle fiber, 6–10°C | 0.05       | $1.5 \times 10^8$   | 12         | 0.080     |
|                        |                         |             | (0.375 $\times 10^8$)* | (0.320)*   |
| Hollingworth et al. (1992) | Intact muscle fiber, 16°C | 0.3–0.8   | $0.25 \times 10^8$ | 17         | 0.680     |
| This article          | Cut muscle fiber, 14°C  | 0.5–1       | $0.4 \times 10^8$    |            |           |

Column 1 gives the reference. Column 2 gives the conditions of the measurements. Column 3 gives the concentration of fura-2. Columns 4 and 5 give, respectively, the forward ($k_t$) and backward ($k_{-1}$) rate constants for the reaction between the Ca and fura-2. Column 6 gives the $K_D$, the dissociation constant of the reaction, which is equal to $k_{-1}/k_1$. See text for additional information.

*Since antipyrylazo III is expected to underestimate the amplitude of $\Delta[Ca]$ by a factor of four (Maylie et al., 1987 a, c; Hirota et al., 1989; Konishi and Baylor, 1991; Konishi et al., 1991), these values of $k_1$ and $K_D$ were corrected by multiplication of the corresponding values in the preceding line by 0.25 and 4, respectively.

The delays inside a muscle fiber may actually be greater than these estimates, since the values of $k_1$ and $k_{-1}$ in myoplasm appear to be smaller than those measured in calibration solutions. Klein et al. (1988) compared Ca signals recorded simultaneously from fura-2 and antipyrylazo III inside cut muscle fibers at 6–10°C. Myoplasmic free $[Ca]$ was estimated from the antipyrylazo III signal on the assumptions that all the indicator inside a fiber is able to react with Ca as it does in calibration solutions and that the speed of this reaction is instantaneous. Eq. 10 was then used to derive the time course and amplitude of $[Cafura-2]$ from $[Ca]$ with the values of $k_1$ and $k_{-1}$ adjusted to give a least-squares fit. They obtained mean values $k_1 = 1.49 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 11.9 \text{s}^{-1}$, which gives $K_D = 0.080 \mu\text{M}$. If antipyrylazo III underestimates the amplitude of the [Ca] signal, as seems likely (Maylie et al., 1987a, c; Hirota et al., 1989; Konishi and Baylor, 1991; Konishi et al.,
1991), the estimated value of $k_1$ should be reduced accordingly but $k_{-1}$ would remain unchanged.

Baylor and Hollingworth (1988) also compared Ca signals recorded from fura-2 and antipyrylazo III, in this case in intact fibers at 16°C. Hollingworth et al. (1992) give mean values $k_1 = 0.25 \times 10^8 \text{M}^{-1}\text{s}^{-1}$, $k_{-1} = 17 \text{s}^{-1}$, and $K_D = 0.680 \mu\text{M}$ (Table II), on the assumption that the actual change in myoplasmic free [Ca] was equal to four times the value estimated with antipyrylazo III. If the value of $k_1$ obtained by Klein et al. (1988) is divided by four to allow for the same expected calibration inaccuracy with antipyrylazo III, their estimates would give $k_1 = 0.375 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ and $K_D = 0.320 \mu\text{M}$ (values marked with an asterisk in Table II).

The above estimates of $k_1$ inside a muscle fiber vary between $0.25 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ and $1.49 \times 10^8 \text{M}^{-1}\text{s}^{-1}$. Even with the smallest value of $k_1$, Eq. 13 indicates that $\Delta [\text{Cafura-2}]$ is expected to track SR Ca release with a delay <40 ms if [fura-2] ≥ 1 mM.

In Fig. 3, the value of myoplasmic free [Ca] before stimulation should have been given by

$$[\text{Ca}] = \frac{[\text{Cafura-2}]}{[\text{fura-2}] - [\text{Cafura-2}]}$$

(14)

If the value of resting $[\text{Cafura-2}]/[\text{fura-2}]$ at the optical site was equal to 0.25, the same as the value in the end pool solutions, the value of resting free [Ca] should have been $0.33K_D$.

Soon after the action potential, when the values of [Ca] and [Cafura-2] had reached new steady levels, [Cafura-2] was estimated to increase 0.213 mM from its resting value of 4.927/4 = 1.232 mM. According to Eq. 14, free [Ca] should have increased by only a small amount, from 0.33$K_D$ to 0.41$K_D$. Consequently, it seems safe to conclude in Fig. 3 that the intrinsic myoplasmic Ca buffers complexed only a small amount of the Ca that was released from the SR and that fura-2 complexed most of the Ca.

The $d\Delta[\text{Cafura-2}]/dt$ signal is shown at the bottom of Fig. 3. It represents an estimate of the rate of Ca release from the SR. Although the trace is noisy, its main features are readily distinguishable. The signal increases soon after the action potential and is brief; it reaches its half-peak value 1.9 ms after that of the action potential and has a half-width of 2.5 ms. The peak value of the signal is ~ 82 $\mu\text{M}/\text{ms}$.

Traces similar to those in Fig. 3 were obtained at regular intervals during the period when fura-2 was diffusing from the end pool solutions into the optical site. Fig. 4, A and B, shows the values of $\Delta[\text{Cafura-2}]$ and $d\Delta[\text{Cafura-2}]/dt$ after a single action potential plotted as a function of the resting concentration of fura-2. At concentrations <1 mM, the amplitude of both signals increased with resting [fura-2] as more indicator became available to complex the Ca that was released from the SR into the myoplasm. From 1 to 2 mM resting [fura-2], the values of $\Delta[\text{Cafura-2}]$ and $d\Delta[\text{Cafura-2}]/dt$ increased slightly, with maximal plateau values of ~ 240 $\mu\text{M}$ and ~110 $\mu\text{M}/\text{ms}$, respectively. Between 3 and 4 mM, both signals showed slight decreases. At the largest values of resting [fura-2], 3.5–3.8 mM, the peak value of $d\Delta[\text{Cafura-2}]/dt$ was ~ 80 $\mu\text{M}/\text{ms}$ (cf. the $d\Delta[\text{Cafura-2}]/dt$ trace in Fig. 3 at 3.7 mM resting [fura-2]).
The changes in the amplitude of the Δ[Cafura-2] and dΔ[Cafura-2]/dt signals in Fig. 4, A and B, were accompanied by only slight changes in the waveform of dΔ[Cafura-2]/dt: its time to half-peak (after that of the action potential) progressively decreased from ~2.0 to ~1.8 ms during the course of the experiment and its half-width increased from ~1.9 to ~2.7 ms (not shown).

Fig. 4 C shows a plot of the plateau values of Δ[Cafura-2] measured at the end of a train of 30–50 action potentials. As mentioned above, when the value of resting [fura-2] is less than the SR Ca content (expressed as myoplasmic concentration), the

**Figure 4.** Effect of resting [fura-2] on the changes in [Cafura-2] measured after a single action potential and after a tetanus. The filled circles in A and B show, respectively, the peak amplitudes of Δ[Cafura-2] and dΔ[Cafura-2]/dt measured after a single action potential plotted as a function of resting [fura-2]. The filled circles in C show the amplitude of Δ[Cafura-2] measured at the end of a train of 30–50 action potentials plotted as a function of resting [fura-2]. The straight line represents Δ[Cafura-2] = [fura-2]. From the experiment illustrated in Figs. 1–3. In each panel, a vertical tick marks the point taken from the run illustrated in Figs. 2 (ΔA traces) and 3.
The plateau value of $\Delta[C_{\text{fura-2}}]$ is approximately equal to resting [fura-2], represented by the line in Fig. 4 C. It is only when resting [fura-2] is greater than the SR Ca content that the value of $\Delta[C_{\text{fura-2}}]$ can be used to estimate SR Ca content. In Fig. 4 C, the value of $\Delta[C_{\text{fura-2}}]$ progressively increased from a value of 1,253 μM with resting [fura-2] = 1.736 mM to a value of 2,096 μM with resting [fura-2] = 3.740 mM. These results suggest that, in this experiment, the Ca content of the SR progressively increased as the resting concentration of fura-2 at the optical site increased from 1.7 to 3.7 mM. A possible explanation for this increase is that the SR is able to accumulate Ca from the myoplasm of a fiber that has been equilibrated with an end pool solution in which [fura-2] is equal to several millimolar and $[C_{\text{fura-2}}]/[\text{fura-2}] = 0.25$.

Changes in $[C_{\text{fura-2}}]$ Produced by a Single Action Potential and by a Tetanus in a Fiber Exposed to 8 mM Fura-2 + 1 mM Ca in the End Pools

In the experiment illustrated in Figs. 1–4, rather large values of $\Delta [C_{\text{fura-2}}]/dt$ were observed with as much as 3.8 mM fura-2 at the optical site. Since the amplitude of both the $\Delta [C_{\text{fura-2}}]$ and $\Delta [C_{\text{fura-2}}]/dt$ signals showed a tendency to decrease at resting concentrations of fura-2 above 3 mM (Fig. 4, A and B), an experiment was carried out with a larger concentration of fura-2 in the end pools, 8 mM fura-2 with 1 mM Ca. In this experiment, $[C_{\text{fura-2}}]/[\text{fura-2}] = 0.25$ in the end pool solutions was decreased from the value of 0.25 used in Figs. 1–4 to a value of 0.125. This was done in an attempt to reduce the progressive increase in SR Ca content that appeared to accompany the increase in resting fura-2 concentration in Fig. 4 C. We should add, however, that we have no direct evidence to support the idea that changes in the value of $[C_{\text{fura-2}}]/[\text{fura-2}]$ in the end pool solutions between 0.125 and 0.25 affect SR Ca content.

Fig. 5 B shows the time course of indicator diffusion, presented with the same format used for Fig. 1 A. Two vertical ticks mark the times when the two sets of records in Fig. 5 A were taken, with 2.706 and 5.667 mM fura-2. The two action potential records (top traces) are very similar, although the one taken with the larger resting concentration of fura-2 had a slightly smaller amplitude (by 4 mV) and a slightly longer half-width (by 0.4 ms).

The other traces in Fig. 5 A show $\Delta[C_{\text{fura-2}}]$ (the middle two superimposed traces) and $\Delta[C_{\text{fura-2}}]/dt$ (the bottom two traces). Within each pair, the signals obtained with the larger resting concentration of fura-2 had the smaller amplitude.

The traces in Fig. 5 A show that, in this experiment, the main effect of an increase in the resting concentration of fura-2 from 2.7 to 5.7 mM was to decrease the amplitude of the $\Delta[C_{\text{fura-2}}]$ and $\Delta[C_{\text{fura-2}}]/dt$ signals by about half. There was little effect on the action potential or on the temporal waveform of the $\Delta[C_{\text{fura-2}}]$ and $\Delta[C_{\text{fura-2}}]/dt$ signals. The time to half-peak of the $\Delta[C_{\text{fura-2}}]/dt$ signal, after that of the action potential, increased from 1.9 to 2.3 ms and its half-width increased from 2.5 to 2.6 ms.

Fig. 6, A and B, shows the effect of resting [fura-2] on $\Delta[C_{\text{fura-2}}]$ and $\Delta[C_{\text{fura-2}}]/dt$ in the experiment in Fig. 5. The amplitude of both signals increased as the resting concentration of fura-2 at the optical site increased from 0 to 2 mM. Between 2 and 3–3.5 mM, the amplitudes remained relatively constant at about...
Δ[Cafura-2] = 350 μM and dΔ[Cafura-2]/dt = 140 μM/ms. As the concentration of indicator increased further, both amplitudes decreased. These changes in the Cafura-2 signals were accompanied by only small changes in the waveform of dΔ[Cafura-2]/dt: its time to half-peak (after that of the action potential) progressively increased from ~1.8 to ~2.2 ms during the course of the experiment and its

![Figure 5](image)

**Figure 5.** Two sets of active signals (A) and the indicator diffusion curve (B) from an experiment in which 8 mM [fura-2] + 1 mM Ca was used in the end pool solutions. (A) The top two superimposed traces show the action potentials measured when the values of resting [fura-2] at the optical site were 2.706 and 5.667 mM. The amplitude of the second action potential (obtained with 5.667 mM [fura-2]) was 4 mV less than that of the first and its duration was 0.4 ms longer. The next two superimposed traces show Δ[Cafura-2] from the same runs. The amplitude of the trace obtained with 2.706 mM [fura-2] was almost twice as large as that obtained with 5.667 mM indicator. The bottom two traces show dΔ[Cafura-2]/dt, with the trace obtained with 2.706 mM [fura-2] on top. (B) The filled circles show the value of resting [fura-2] at the optical recording site, obtained from indicator-related A(420), plotted as a function of time after the addition of indicator to the end pool solutions. The x’s show values of resting [fura-2] estimated from measurements of ΔA(420) and ΔF/F. The curve shows a least-squares fit of Eqs. 6 and 8 in Maylie et al. (1987b) to the filled circles; D = 0.35 x 10^{-6} cm²/s and (R + 1) = 1.01. The two vertical ticks indicate when the records in A were taken. Fiber reference, 615921; sarcomere spacing, 3.8 μm. Range of values, beginning to end of experiment: fiber diameter, 121–114 μm; holding current, −65 to −98 nA; action potential amplitude, 133–125 mV. Fura-2 was added to the end pool solutions 48 min after saponin treatment of the end pool segments.

half-width increased from ~2.5 to ~3.0 ms (not shown). Some of the decrease in the amplitude of the Cafura-2 signals from 5 to 6 mM fura-2 is probably due to a decrease in the Ca content of the SR, as will now be described.

The filled circles in Fig. 6 C show the plateau values of Δ[Cafura-2] elicited by a train of 30–50 action potentials. These values were relatively stable at 2,800–2,900
µM as resting [fura-2] increased from 3.4 to 4.8 mM. Thereafter, they progressively decreased as resting [fura-2] increased. Part of this progressive decrease appears to be due to incomplete SR Ca depletion. At values of resting [fura-2] > 5 mM, the amplitude of the dΔ[Cafura-2]/dt waveform progressively decreased (Fig. 6 B), indicating that the rate of SR Ca release decreased. As a result, Δ[Cafura-2] did not reach a plateau level during a train of 50 action potentials. Since longer periods of stimulation were not used, it was necessary to estimate the plateau level of Δ[Cafura-2] that would be expected at the end of a
fully depleting train of action potentials. The final time course of the $\Delta$[Ca$^{2+}$] signal during a train of 50 action potentials was fitted with a decreasing exponential function plus a constant and the constant was used for the estimate, plotted as ×'s in Fig. 6 C.

The ×’s in Fig. 6 C probably represent reliable estimates of the Ca content of the SR for resting [fura-2] ≥ 5.334 mM. Thus, the Ca content of the SR appears to have progressively decreased as the resting concentration of fura-2 became > 5 mM. A similar decrease was observed at large concentrations of fura-2 in the experiment in Figs. 9–11 (not shown) and in the experiments illustrated in Figs. 3 B and 5 B in Jong et al. (1993). The reason for the decrease is not known.

Although the SR Ca content in Fig. 6 C appeared to decrease as resting [fura-2] became > 5 mM, the magnitude of the decrease was not as marked as the decreases in [Ca$^{2+}$] and $\Delta$[Ca$^{2+}$]/$dt$ in Fig. 6, A and B, respectively. Thus, fura-2 seems to have had an inhibitory effect on SR Ca release that cannot be explained solely by a reduction of SR Ca content.

**Changes in [Ca$^{2+}$] Produced by a Single Action Potential and by a Tetanus in a Fiber Exposed to 8 mM Fura-2 + 0 mM Ca in the End Pools**

In the experiment in Fig. 6, A and B, the values of $\Delta$[Ca$^{2+}$] and $\Delta$[Ca$^{2+}$]/$dt$ progressively increased as the resting concentration of fura-2 at the optical site increased from 0 to ~3 mM. If the resting value of [Ca$^{2+}$/fura-2] remained constant during this period and equal to its value in the end pool solutions, [Ca$^{2+}$] should have increased from 0 to ~0.375 mM. If the SR Ca pump had been able to reduce free [Ca$^{2+}$] below the level set by [Ca$^{2+}$/fura-2] in the end pool solutions, 0.125, the Ca$^{2+}$ that diffused into the optical site might have provided a source of Ca to be pumped into the SR. Thus, some of the increase in $\Delta$[Ca$^{2+}$] and $\Delta$[Ca$^{2+}$]/$dt$ from 0 to 3 mM fura-2 in Fig. 6, A and B, might have been due to an increase in SR Ca content rather than to an increase in SR Ca permeability. Unfortunately, our method does not permit a reliable estimate of SR Ca content until the value of the resting concentration of fura-2 exceeds that of SR content. It was therefore of interest to try to minimize any additional uptake of Ca by the SR as the value of resting [fura-2] increased from 0 to 3 mM. For this purpose, an end pool solution that contained fura-2 without added Ca was used.

Fig. 7 shows the results of an experiment in which 8 mM fura-2 (without Ca) was present in the end pools for 67.6 min. Then, the end pools were rinsed with fura-2-free solution, which reduced the final concentration to 0.16 mM. In each panel of Fig. 7, filled circles show data obtained during the period when 8 mM fura-2 was present in the end pools and open circles show data obtained after its nominal removal.

Fig. 7 D shows the time course of indicator concentration at the optical site. The theoretical curve, which was fitted to the filled circles, provides a good fit to the data during the period when indicator concentration was increasing and initially decreasing. It fails to fit the data at late times when some of the indicator appeared to be unable to diffuse freely out of the fiber, similar to the behavior of arsenazo III (Maylie et al., 1987b) and antipyrylazo III (Maylie et al., 1987c).

Fig. 7 A shows the values of $\Delta$[Ca$^{2+}$] obtained after a single action potential,
FIGURE 7. Effect of resting [fura-2] on the changes in [Ca^{2+}]-fura-2 measured after a single action potential and after a tetanus, from a fiber in which 8 mM fura-2 (without added Ca) was present in the end pool solutions for the first 67.6 min of the experiment and then was nominally removed. Filled circles denote data obtained during the period when fura-2 was present in the end pool solutions and open circles denote data obtained after its removal. The format of A–C is the same as that of Figs. 4 and 6. The format of D is similar to that of Figs. 1A and 5B. The curve in D was fitted to the filled circles, which gave $D = 0.49 \times 10^{-6}$ cm$^2$/s and $(R + 1) = 0.95$. The same parameters were then used to calculate the time course of resting [fura-2] at the optical site after its removal from the end pools. For the calculation, the value of [fura-2] in the end pools after removal was taken to be 0.16 mM, as estimated from a measurement of the solution's 420-nm absorbance at the end of the experiment. Fiber reference, 619921; sarcomere spacing, 4.2 m. Range of values, beginning to end of experiment: fiber diameter, 94–86 m; holding current, −37 to −52 nA; action potential amplitude, 136–129 mV. Fura-2 was added to the end pool solutions 44 min after saponin treatment of the end pool segments.
plotted as a function of resting [fura-2]. During the period when resting [fura-2] increased with time at the optical site (filled circles and the open circle at the far right), the value of $\Delta$[Cafura-2] first increased. Between 1 and 3 mM resting [fura-2], $\Delta$[Cafura-2] was relatively constant at 280–290 $\mu$M and, at higher concentrations, it progressively decreased. When the value of resting [fura-2] reached 6.005 mM, the value of $\Delta$[Cafura-2] had decreased to slightly more than half of its maximal value (open circle at far right). After that measurement was made, the resting concentration of fura-2 at the optical site decreased and the $\Delta$[Cafura-2] signal increased (other open circles).

Fig. 7 B shows the peak values of $\frac{d\Delta\text{[Cafura-2]}}{dt}$ plotted as a function of resting [fura-2]. The relations shown by the filled and open circles are qualitatively similar to those in Fig. 7 A. As fura-2 diffused into the fiber (filled circles), the value of $\frac{d\Delta\text{[Cafura-2]}}{dt}$ reached a maximum of $\sim 130$ $\mu$M/ms at resting [fura-2] = 1–3 mM and then declined at larger values of resting [fura-2]. As fura-2 left the fiber (open circles), $\frac{d\Delta\text{[Cafura-2]}}{dt}$ reached a maximum of 160–170 $\mu$M/ms at resting [fura-2] = 1.2–2 mM.

The open and filled circles in Fig. 7 A and B, do not overlap one another, showing that the relation between $\Delta$[Cafura-2] or $\frac{d\Delta\text{[Cafura-2]}}{dt}$ and resting [fura-2] underwent hysteresis. The hysteresis between 4 and 6 mM resting [fura-2] appears to have been due to hysteresis in SR Ca permeability since, according to Fig. 7 C, SR Ca content appears to have been relatively constant without any hysteresis in its fura-2 dependence.

Fig. 7 C shows the plateau value of $\Delta$[Cafura-2] produced by a fully depleting tetanus. As fura-2 diffused into the optical site (filled circles), the value of $\Delta$[Cafura-2] remained relatively constant at 2,000–2,100 $\mu$M from 2.7 to 4 mM resting [fura-2]. From 4 to 6 mM resting [fura-2], the value of $\Delta$[Cafura-2] decreased, reversibly, by 10–15%. As the resting concentration of fura-2 decreased from 4 to 2.6 mM (open circles), the value of $\Delta$[Cafura-2] increased from 2,100 to 2,400 $\mu$M. The three open circles plotted at 1.1–1.2 mM resting [fura-2], obtained at the end of the experiment, almost overlap the line, which represents $\Delta$[Cafura-2] = resting [fura-2]. This shows that almost all the resting fura-2 was able to react with Ca, although, according to Fig. 7 D, it was unable to diffuse freely away from the optical site.

In the experiment in Fig. 7, the values of $\Delta$[Cafura-2] (A) and $\frac{d\Delta\text{[Cafura-2]}}{dt}$ (B) that were obtained with resting [fura-2] $\leq$ 2 mM were larger at the end of the experiment (open circles) than at the beginning (filled circles). Although the reason for this is unknown, a possible contributing factor is that the SR Ca content increased toward the end of the experiment. This explanation would be consistent with the increase in the plateau values of $\Delta$[Cafura-2] observed in Fig. 7 C (open circles) as the resting concentration of fura-2 decreased from 4 to 2.6 mM. Unfortunately, the SR Ca content could not be estimated later in the experiment, when resting [fura-2] was $< 2.5$ mM, because the resting concentration of fura-2 was insufficient to complex the Ca released from the SR. If the Ca content of the SR did, in fact, increase toward the end of the experiment, the Ca must somehow have entered the fiber from the external solution since no Ca was added to the end pool solutions.

Changes in several other parameters were observed during the experiment in Fig. 7. In general, these changes were minor and occurred in a progressive manner that
appeared to be related to the time that had elapsed after the experiment was started rather than to the resting concentration of fura-2 present at the optical site: (a) The amplitude of the action potential decreased steadily from 136 to 129 mV. (b) The holding current changed steadily from -37 to -52 nA. (c) The time to half-peak of the $\Delta[A_{\text{Ca}}]/dt$ signal (after that of the action potential) increased ~0.2 ms from an initial value of ~1.5 ms. (d) The half-width of the $\Delta[A_{\text{Ca}}]/dt$ signal increased by ~0.5 ms from an initial value of ~2 ms. It seems likely that some of these changes, possibly also including the increase in SR Ca content toward the end of the experiment, may have been due to fiber run down.

### Table III

| Fiber Reference | $[\text{Cafura-2}]$ (mM) | $[\text{Fura-2}]$ (mM) | $\Delta[A_{\text{Ca}}]/dt$ (\(\mu\text{M}/\text{ms}\)) | $\Delta[A_{\text{Ca}}]/dt$ (\(\%\text{ms}\)) | $\Delta[A_{\text{Ca}}]/dt$ (\(\mu\text{M}/\text{ms}\)) | $\Delta[A_{\text{Ca}}]/dt$ (\(\%\text{ms}\)) | $\Delta[A_{\text{Ca}}]/dt$ (\(\mu\text{M}/\text{ms}\)) | $\Delta[A_{\text{Ca}}]/dt$ (\(\%\text{ms}\)) | SR Ca Content (\(\mu\text{M}\)) |
|-----------------|--------------------------|------------------------|-------------------------------------|------------------------|-------------------------------------|------------------------|-------------------------------------|------------------------|--------------------------|
| 318921 (1/4)    | 2.0-4.0                  |                        | 90                                  | 4.86                   | 92                                  | 90                     | 4.86                                | 92                     | 4.86                     |
| 323921 (1/4)    | 4.0-5.0                  |                        | 111                                 | 5.27                   | 112                                 | 5.27                   | 111                                 | 5.27                   | 112                      |
| 615921 (1/8)    | 2.0-4.0                  |                        | 139                                 | 5.27                   | 112                                 | 4.24                   | 71                                  | 2.89                   | 2,852-2,576              |
| 616921 (1/8)    | 4.0-5.0                  |                        | 173                                 | 7.80                   | 136                                 | 7.61                   | 118                                 | 4.69                   | 2,416-2,566              |
| 618922 (2/8)    | 5.0-6.0                  |                        | 110                                 | 6.10                   | 100                                 | 5.29                   | 43                                  | 3.70                   | 2,069-1,306              |
| 619921 (0/8)    |                          |                        | 128                                 | 6.63                   | 115                                 | 5.96                   | 77                                  | 4.16                   | 2,050-1,933              |
| Mean            |                          |                        | 138                                 | 6.45                   | 116                                 | 5.78                   | 77                                  | 3.86                   |                          |
| SEM             |                          |                        | 13                                  | 0.55                   | 7                                   | 0.71                   | 15                                  | 0.38                   |                          |

Column 1 gives the fiber reference with the values of $[\text{Cafura-2}]$ and $[\text{Fura-2}]$ in millimolar in the end pool solutions, separated by a slash mark, given in parentheses. Column 2 gives the peak values of $\Delta[A_{\text{Ca}}]/dt$ obtained with 2.0-4.0 mM fura-2; column 3 gives the peak values after correction for SR Ca depletion, as described in the text. Columns 4-5 and 6-7 are similar in format to columns 2-3 except that [fura-2] = 4.0-5.0 and 5.0-6.0 mM, respectively. Column 8 gives the range of values of SR Ca content, expressed in terms of total myoplasmic concentration, that were used for the depletion corrections in columns 3, 5, and 7. Fibers 318921, 615921, and 619921 were studied with fura-2 alone and fibers 323921, 616921, and 618922 were studied with fura-2 plus PDAA. Since fibers 318921 and 323921 were studied with 4 mM fura-2 and 1 mM Ca in the end pool solutions, the concentration of fura-2 at the optical site did not get sufficiently large for entries to be obtained for columns 4-7; consequently, the values in columns 2 and 3 for these fibers were not included in the calculation of the mean and SEM. The values for fiber 619921 were obtained during the period when fura-2 was diffusing into the optical site (filled circles in Fig. 7).

**Summary of the Effect of 2-6 mM Fura-2 on $\Delta[A_{\text{Ca}}]/dt$ Produced by a Single Action Potential**

Table III shows the effect of resting fura-2 concentration on the peak rate of SR Ca release elicited by a single action potential. The release rates were estimated from $\Delta[A_{\text{Ca}}]/dt$ directly and therefore do not depend on model calculations. Fibers 318921, 615921, and 619921 contained only fura-2. The other three fibers contained a mixture of fura-2 plus PDAA, as described below.

Column 1 of Table III gives the fiber reference with the values of $[\text{Cafura-2}]$ and $[\text{Fura-2}]$ in the end pool solutions given in parentheses. Column 2 gives the mean
peak value of $dA[Ca^{2+}]_r/dt$ obtained from each fiber with 2--4 mM resting [fura-2] at the optical recording site.

Column 3 of Table III is similar to column 2 except that the peak values of $dA[Ca^{2+}]_r/dt$ have been corrected for SR Ca depletion and expressed in units of percent per millisecond, a procedure introduced by Jacquemond et al. (1991). For the correction, the value of $dA[Ca^{2+}]_r/dt$ was divided by the value of readily releasable [Ca] remaining inside the SR, which is given by the concentration of readily releasable Ca inside the SR before stimulation minus the concentration of Ca that had already been released (all expressed in terms of myoplasmic concentration); the final result was multiplied by 100 to give units of percent per millisecond. The concentration of readily releasable Ca inside the SR before stimulation was taken to equal the value of $A[Ca]_r$ that was measured after a depleting train of action potentials (see Figs. 4 C, 6 C, and 7 C). Columns 4--5 and 6--7 of Table III are similar to 2--3 except that resting [fura-2] = 4--5 and 5--6 mM, respectively.

Column 8 gives the range of values of SR Ca content, expressed as myoplasmic concentration. The variation of values among different fibers prevents a determination of the relation between SR Ca content and the value of $[Ca^{2+}]_r/[Ca^{2+}]_t$ in the end pool solutions, which varied from 0 to 0.25.

According to the mean values of columns 2--3, 4--5, and 6--7 in Table III, 4--5 and 5--6 mM resting [fura-2] reduced $dA[Ca^{2+}]_r/dt$ to 0.84--0.90 and 0.56--0.60 times that observed with 2--4 mM resting [fura-2]. A decrease in $dA[Ca^{2+}]_r/dt$ with increasing fura-2 concentration is consistent with the idea that fura-2 may block Ca-induced Ca release. Alternatively, it is also consistent with the possibility that a large concentration of fura-2 might have a pharmacological effect to decrease SR Ca release.

SR Ca Release Calculated from PDAA Ca Transients Elicited by 1 and 10 Action Potentials

In the action potential experiments illustrated in Figs. 1--7, fura-2 was used without any other Ca indicator so that the interpretation of the fura-2 signals would be as direct as possible. A serious limitation of these experiments is that they provide no information about myoplasmic free [Ca] transients in the absence of fura-2. Consequently, there is no way to decide whether the peak rate of SR Ca release with action potential stimulation actually increased or decreased when resting [fura-2] was increased from 0 to 2--3 mM.

Baylor and Hollingworth (1988) and Jacquemond et al. (1991) used antipyrpyrlyazo III to measure $\Delta[Ca]$ signals before fura-2 was injected into a fiber. Because of difficulties associated with the calibration of antipyrpyrlyazo III signals inside muscle fibers (Maylie et al., 1987c), we have used one of the new purpurate indicators, PDAA, to measure $\Delta[Ca]$ (Hirota et al., 1989). As discussed by Hirota et al. (1989), these purpurate indicators appear to provide the most reliable direct estimates that are presently available of both the amplitude and time course of free $\Delta[Ca]$ after an action potential in a twitch muscle fiber.

In our experiments with both PDAA and fura-2, PDAA was always introduced into the end pools first. When its concentration at the optical site had reached 2.1--2.5 mM, measurements of action potential-stimulated signals were made. Then, a
solution containing fura-2 plus PDAA was introduced into the end pools. Fig. 8 shows results obtained with PDAA before fura-2 was applied.

The top two traces in Fig. 8A show the action potential and the associated change in myoplasmic free [Ca], obtained from the indicator-related $A_\Delta(A(570))$ signal as described in Methods. Model 1 (Table I) was then used to calculate $A_\Delta(Ca_{Trop})$ (the estimated change in the myoplasmic concentration of Ca bound to the Ca-regulatory sites on troponin), $A_\Delta(Ca_{Parv})$ (the estimated change in the myoplasmic concentration of Ca bound to the Ca,Mg sites on parvalbumin; not shown), and $A_\Delta(Ca_T)$ (the estimated change in total myoplasmic Ca, which is equal to $A_\Delta(Ca) + A_\Delta(Ca_{PDAA}) + A_\Delta(Ca_{Trop}) + A_\Delta(Ca_{Parv})$). The computational procedure was the same as that used by Baylor et al. (1983) except that the myoplasmic concentration of Ca,Mg sites on parvalbumin was taken to be 1.5 mM (model 1, Table I) rather than 1 mM. The $A_\Delta(Ca_{Trop})$ and $A_\Delta(Ca_T)$ traces are shown superimposed in Fig. 8A, just above the $dA_\Delta(Ca_T)/dt$ signal at the bottom.

In Fig. 8A, the $A_\Delta(Ca)$ signal had a peak value of 14.3 μM, a time to half-peak of 2.6
ms (after that of the action potential), and a half-width of 7.8 ms. The calculated peak values of $\Delta\text{[CaTrop]}$ and $\Delta\text{[CaT]}$ were 197 and 308 $\mu$M, respectively. The $\text{d}\Delta\text{[CaT]}/\text{d}t$ signal had a peak value of 131 $\mu$M/ms, a time to half-peak of 1.9 ms, and a half-width of 2.2 ms.

PDAA Ca transients were measured in three fibers just before the addition of fura-2 to the end pools. On average, with 2.3–2.8 mM PDAA, the $\Delta\text{[Ca]}$ signal had a peak value of 14.4 $\mu$M, a time to half-peak of 2.5 ms, and a half-width of 6.9 ms; these values are similar to those obtained by Hirota et al. (1989).

The calculated $\Delta\text{[CaTrop]}$ and $\Delta\text{[CaT]}$ signals in the three fibers had mean peak values of 193 and 294 $\mu$M, respectively. The $\text{d}\Delta\text{[CaT]}/\text{d}t$ signal had a mean peak value of 138 $\mu$M/ms, a time to half-peak (after that of the action potential) of 1.8 ms, and a half-width of 2.1 ms. These values are similar to those obtained by Maylie et al. (1987a) with tetramethylmurexide (for the myoplasmic component of the signal) and by Maylie et al. (1987c) with antipyrylazo III (high Ca calibration); $\Delta\text{[CaTrop]}$ and $\Delta\text{[CaT]}$ signals were not calculated by Hirota et al. (1989).

Fig. 8 B shows traces obtained with a train of 10 action potentials at 50 Hz. The format is similar to that in Fig. 8 A except that the time base is different. As observed in Fig. 8 A, the $\Delta\text{[CaT]}$ signal rapidly increased after the first action potential, due to Ca complexation by both the Ca-regulatory sites on troponin and the Ca,Mg-free sites on parvalbumin. The peak value of myoplasmic free $\Delta\text{[Ca]}$ after the first action potential was greater than that observed after the second and subsequent action potentials (Hirota et al., 1989). Nonetheless, the value of $\Delta\text{[Ca]}$ during the train was sufficiently high that the amount of Ca complexed by troponin remained approximately constant. Although the values of $\Delta\text{[Ca]}$, $\Delta\text{[CaPDAA]}$, and $\Delta\text{[CaTrop]}$ were relatively constant after the first one to two action potentials, a small progressive increase in $\Delta\text{[CaT]}$ occurred. This small increase was due to Ca complexation by Ca,Mg sites on parvalbumin that became available as Mg slowly dissociated from them (not shown).

For the purposes of this article, the most interesting feature of the traces in Fig. 8 B is the fivefold reduction of the amplitude of the $\text{d}\Delta\text{[CaT]}/\text{d}t$ signal from the first to the second action potential. This decrease is similar to that observed by Baylor et al. (1983), who used arsenazo III to monitor $\Delta\text{[Ca]}$, and Maylie et al. (1987c) and Baylor and Hollingworth (1988), who used antipyrylazo III to monitor $\Delta\text{[Ca]}$. It is probably due to Ca inactivation of Ca release.

SR Ca Release Estimated from PDAA Ca Transients and $\Delta\text{[Cafura-2]}$

Fig. 9 shows the time course of the resting concentrations of PDAA and fura-2 at the optical recording site in an experiment with both indicators. The filled squares show the concentration of PDAA plotted as a function of time after 2.923 mM PDAA was introduced into the end pools. The open squares and filled circles show, respectively, the resting concentrations of PDAA and fura-2 beginning 46 min later, when the end pool solutions were changed to 2.446 mM PDAA plus 8 mM fura-2 with 2 mM Ca. The $\times$ represents an estimate of the resting concentration of fura-2 that was obtained from $\Delta A(410)$ and $\Delta F/F$. Its value is in agreement with the filled circles, indicating that the value of indicator-related $A(410)$ provided a reliable estimate of the resting
concentration of fura-2 in the presence of PDAA. The two continuous curves were calculated from the one-dimensional diffusion equation with linear myoplasmic binding, as was done in Figs. 1 A, 5 B, and 7 D. The curve plotted from 0 to 46 min was fitted to the filled squares (PDAA) and the other curve was fitted to the filled circles (fura-2).

The parameters associated with the PDAA curve were \( \frac{D}{(R + 1)} = 0.96 \times 10^{-6} \text{ cm}^2/\text{s}, \ (R + 1) = 0.93, \) and \( D = 0.90 \times 10^{-6} \text{ cm}^2/\text{s} \). In a total of eight fibers from *Rana temporaria*, three studied with action potential solutions and five with voltage-clamp solutions (*Jong et al., 1993*), the average values were \( \frac{D}{(R + 1)} = 0.86 \times 10^{-6} \text{ cm}^2/\text{s} \) (SEM, \( 0.03 \times 10^{-6} \text{ cm}^2/\text{s} \)), \( (R + 1) = 0.99 \) (SEM, 0.02), and \( D = 0.85 \times 10^{-6} \text{ cm}^2/\text{s} \) (SEM, 0.03). The value of \( \frac{D}{(R + 1)} \) is slightly less than that reported by *Hirota et al. (1989)*, \( 1.07 \times 10^{-6} \text{ cm}^2/\text{s} \) (SEM, \( 0.06 \times 10^{-6} \text{ cm}^2/\text{s} \)), which may be due, at least in part, to the difference in temperature of the two sets of measurements (14°C in our experiments and 16°C in the experiments of *Hirota et al., 1989*). The value of \( (R + 1) \) is less than that obtained by *Hirota et al. (1989)*, 1.23 (SEM, 0.04). The reason for this difference, which is statistically significant, is not known. The time course of fura-2 diffusion was described above in connection with Fig. 1 A.

**Fig. 9.** Time course of the resting concentrations of PDAA and fura-2 at the optical recording site plotted as a function of time after the introduction of 2.923 mM PDAA into the end pools. 46 min later, the end pool solution was exchanged for one that contained 2.446 mM PDAA plus 8 mM fura-2T with 2 mM Ca. The filled squares show [PDAA] at the optical site during the period when only PDAA was present in the end pool solutions; the open squares show [PDAA] after the solution change, when [PDAA] was reduced to 0.84 times its initial value and fura-2 was added. The curve represents a least-squares fit of Eqs. 6 and 8 in *Maylie et al. (1987b)* to the filled squares, with \( D = 0.90 \times 10^{-6} \text{ cm}^2/\text{s} \) and \( (R + 1) = 0.93 \). It is plotted during the period when PDAA without fura-2 was present in the end pools. The filled circles show resting [fura-2] at the optical site, estimated from fura-2-related \( A(410) \). The curve represents a least-squares fit of Eqs. 6 and 8 in *Maylie et al. (1987b)*, with \( D = 0.70 \times 10^{-6} \text{ cm}^2/\text{s} \) and \( (R + 1) = 1.14 \). The \( \times \) gives the value of resting [fura-2] estimated from measurements of \( \Delta A(410) \) and \( \Delta F/F \). The four vertical ticks show when the records in Fig. 10 were obtained. Fiber reference, 618922; sarcomere spacing, 4.1 \( \mu \text{m} \). Range of values, beginning to end of experiment: fiber diameter, 111–115 \( \mu \text{m} \); holding current, \(-36 \) to \(-58 \) nA; action potential amplitude, 130–124 mV. PDAA was initially introduced into the end pools 22 min after saponin treatment of the end pool segments and fura-2 was introduced 46 min later.
FIGURE 10. Ca signals estimated with PDAA and fura-2, from the experiment in Fig. 9. (A) The top trace shows the action potential. The next three traces show, in sequence, Δ[Ca], Δ[CaT], and dΔ[CaT]/dt. Δ[Ca] was estimated from PDAA-related ΔA(570). Δ[CaT] was calculated from Δ[Ca] with model 1, Table I. At the optical site, [PDAA] = 2.200 mM and [fura-2] = 0 mM. (B) The traces are similar to those in A except that both PDAA (2.524 mM) and fura-2 (0.504 mM) were present at the optical site. Δ[Ca], as in A, was estimated from PDAA-related ΔA(570). Δ[Cafura-2] was estimated from fura-2-related ΔA(410) as described in Methods. Δ[CaT] was estimated from Δ[Ca] as in A, and then Δ[Cafura-2] was added to it. The Δ[CaT] and Δ[Cafura-2] traces are shown superimposed. The bottom two traces show dΔ[CaT]/dt and dΔ[Cafura-2]/dt. C and D are similar to B except that the traces were obtained later in the experiment when the resting concentration of fura-2 had increased. In C, [PDAA] = 2.603 mM and [fura-2] = 2.344 mM. In D, [PDAA] = 2.576 mM and [fura-2] = 5.529 mM.
been introduced into the end pools. The first two traces show, respectively, the action potential and the Δ[Ca] signal obtained from the PDAA-related Δ A (570) signal. The Δ[Ca] signal was then used to calculate SR Ca release with model 1 (Table I). The lower two traces show Δ[CaT] and dΔ[CaT]/dt. The peak values of Δ[Ca], Δ[CaT], and dΔ[CaT]/dt were, respectively, 11.2 μM, 272 μM, and 117 μM/ms.

The traces in Fig. 10 B were obtained after the resting concentration of fura-2 at the optical site had reached 0.504 mM. The top trace shows the action potential, which was essentially unchanged from that in Fig. 10 A. The second trace shows the Δ[Ca] signal. The presence of 0.504 mM fura-2 reduced its peak value from 11.2 μM (Fig. 10 A) to 4.0 μM (Fig. 10 B) and shortened its duration, probably because of the Ca-buffering capacity of fura-2. The third row shows the Δ[Ca-fura-2] trace superimposed with Δ[CaT]; Δ[CaT] is equal to the value of Δ[CaT] calculated from the Δ[Ca] trace with model 1 (Table I) plus the value of Δ[Ca-fura-2]. These traces had peak values Δ[Ca-fura-2] = 229 μM and Δ[CaT] = 368 μM. The time derivatives of these traces are shown at the bottom of the figure. The peak values were dΔ[Ca-fura-2]/dt = 84 μM/ms and dΔ[CaT]/dt = 141 μM/ms.

Fig. 10, C and D, shows traces obtained at later times when resting [fura-2] = 2.344 mM (C) and 5.529 mM (D). In both C and D the Δ[Ca] signal was essentially zero, so that the Δ[CaT] signal, at least for the first 5–10 ms, was almost equal to Δ[Ca-fura-2]. A small difference became apparent at later times because the Δ[Ca] signal did not return precisely to the prestimulus baseline. The amplitudes of both the Δ[Ca-fura-2] and dΔ[Ca-fura-2]/dt signals decreased from C to D.

The signal-to-noise ratio associated with the traces of Δ[Ca-fura-2], and consequently of Δ[CaT], in Fig. 10 is smaller than that associated with the Δ[Ca-fura-2] traces in Figs. 3 and 5 A. Part, or perhaps all, of the reason is that half the transmitted light was used for the detection of the Δ A(410) or Δ A(420) signal in fibers with only fura-2 (Figs. 3 and 5 A), whereas one-fourth the light was used in experiments with both fura-2 and PDAA (see Irving et al., 1987, for a description of the apparatus). The source of the quasi-periodic noise in Fig. 10 is not clear. Since it was not synchronized with the 60-Hz line frequency, which was used routinely to synchronize the stimulus for the (first) action potential, there was no easy way to remove it. Estimates of the peak amplitudes of the dΔ[Ca-fura-2]/dt and dΔ[CaT]/dt signals were made with quadratic fits typically to 7–12 sequential points to reduce contributions from the noise.

Fig. 11, A and B, shows the peak values of the Δ[Ca-fura-2] and Δ[CaT] signals (A) and their derivatives (B) plotted as a function of the resting concentration of fura-2. The Δ[Ca-fura-2] results (open circles) are similar to those obtained without PDAA, panels A and B in Figs. 4, 6, and 7, consistent with the idea that PDAA did not affect either SR Ca release or the myoplasmic reaction between Ca and fura-2. The Δ[CaT] results (filled circles) provide additional information about the relation between resting [fura-2] and SR Ca release. As the value of resting [fura-2] increased from 0 to 0.5 mM, the peak values of Δ[CaT] and Δ[CaT]/dt increased by 35 and 21% respectively. As resting [fura-2] increased further, the values of Δ[CaT] and dΔ[CaT]/dt progressively decreased and, at ~4 mM, were approximately equal to the values estimated at 0 mM. As the value of resting [fura-2] increased from 4 to 6 mM, the values of Δ[CaT] and dΔ[CaT]/dt, which were essentially equal to those of
Figure 11. Effect of resting [fura-2] on the Δ[Cₘ] and Δ[Cₐ2] signals and the accompanying amplitude of Δ[Ca], determined after a single action potential, from the experiment in Figs. 9 and 10. (A) The open and filled circles show, respectively, the amplitudes of the Δ[Cₐ2] and Δ[Cₘ] signals after a single action potential, plotted as a function of resting [fura-2]. B shows a plot, similar to that in A, of the peak amplitudes of dΔ[Ca]/dt and dΔ[Cₐ2]/dt after a single action potential. (C) The filled circles show the amplitude of myoplasmic free Δ[Ca] at the time when dΔ[Cₐ2]/dt had reached its peak value, plotted as a function of resting [fura-2]. D is similar to C except that the integral of the Δ[Ca] signal is shown. The integration time extended from the time of stimulation to the time when dΔ[Cₐ2]/dt had reached its peak value. Vertical ticks in each panel mark the points associated with the records in Fig. 10, B–D.
\( \Delta[\text{Ca} \text{-fura-2}] \) and \( \Delta [\text{Ca} \text{-fura-2}]/dt \), decreased dramatically, more so than in either Fig. 6 or 7.

Since some, and possibly all, of the effects of resting [fura-2] on \( \Delta [\text{Ca}_T]/dt \) may be due to the Ca-buffering capacity of fura-2, it was of interest to determine the value of \( \Delta[\text{Ca}] \) at the time that \( \Delta [\text{Ca}_T]/dt \) was at its peak. Fig. 11 C shows these values plotted as a function of resting [fura-2]. The value of \( \Delta[\text{Ca}] \) progressively decreased as the concentration of fura-2 increased. It was half-maximal at \( \sim 0.5 \text{ mM} \) and became indistinguishable from the baseline above 3 mM. This reduction of the bulk myoplasmic \( \Delta[\text{Ca}] \) signal was associated with increases in the \( \Delta[\text{Ca}_T] \) and \( \Delta [\text{Ca}_T]/dt \) signals above the levels observed without fura-2 (Fig. 11, A and B). Once resting [fura-2] was \( \geq 4 \text{ mM} \), the \( \Delta [\text{Ca}_T] \) and \( \Delta [\text{Ca}_T]/dt \) signals showed marked decreases with resting fura-2 concentration, although the amplitude of the \( \Delta[\text{Ca}] \) signal was no more than a small fraction of 1 \( \mu \text{M} \) and was completely obscured by noise.

Since the effects of free Ca on the rate of SR Ca release might be cumulative rather than instantaneous, the \( \Delta[\text{Ca}] \) signal was also integrated from the time of stimulation to the time that \( \Delta [\text{Ca}_T]/dt \) had reached its peak. The filled circles in Fig. 11 D show the integrated values plotted as a function of resting [fura-2]. The points show a progressive decrease with fura-2 concentration, similar to that in Fig. 11 C.

Comparison of Three Different Methods to Estimate SR Ca Release from Ca Transients

In the results described above, SR Ca release was estimated with model 1 (Table I), which is essentially the same as the model used by Hollingworth and Baylor (1988) and Hollingworth et al. (1992). SR Ca release was also estimated with the method used by Jacquemond et al. (1991), model 2 in Table I. There are two main differences between models 1 and 2: (a) the rate constants associated with the binding of Ca to the Ca-regulatory sites on troponin are smaller in model 1 than in model 2 and the corresponding dissociation constants are different, \( K_D = 2 \mu \text{M} \) in model 1 and 7.7 \( \mu \text{M} \) in model 2; and (b) model 2, but not model 1, includes 200 \( \mu \text{M} \) of Ca binding sites associated with the SR Ca pump that are assumed to equilibrate instantaneously with Ca with a relatively high affinity, \( K_D = 1 \mu \text{M} \). Since Jacquemond et al. (1991) estimated \( \Delta[\text{Ca}] \) signals with antipyrylazo III, the amplitude of their \( \Delta[\text{Ca}] \) signals is expected to be only 0.2–0.25 times that estimated with PDAA (Maylie et al., 1987a, c; Hirota et al., 1989; Konishi and Baylor, 1991; Konishi et al., 1991). Consequently, calculations with model 2 were carried out with the unscaled \( \Delta[\text{Ca}] \) signal (model 2:2\( \Delta[\text{Ca}] \)) and with the signal scaled by 0.25 (model 2:0.25\( \Delta[\text{Ca}] \)) to permit a more direct comparison with the results of Jacquemond et al. (1991).

Table IV provides a summary of the effect of 0–4 mM fura-2 on the rate of SR Ca release in three fibers. Column 1 gives the fiber reference with the values of [Ca-fura-2] and [fura-2] in the end pool solutions given in parentheses. Column 2 gives the method that was used to calculate \( \Delta [\text{Ca}_T] \) from the \( \Delta [\text{Ca}] \) waveform. Column 3 gives the peak value of \( \Delta [\text{Ca}_T]/dt \) that was estimated before fura-2 had been introduced into the end pools. A comparison of the values from the three fibers and three methods of calculation shows that model 1 and model 2:0.25\( \Delta[\text{Ca}] \) gave similar peak values of \( \Delta [\text{Ca}_T]/dt \) and that model 2:\( \Delta[\text{Ca}] \) consistently gave larger values. The shape of the \( \Delta [\text{Ca}_T]/dt \) waveform calculated with model 2:\( \Delta[\text{Ca}] \) was surprising...
in that there were two peaks (not shown). Two peaks were also observed in voltage-clamp experiments (Jong et al., 1993). As discussed in Jong et al. (1993), there is reason to doubt the accuracy of the calculations with model 2:Δ[Ca].

Columns 4 and 5 in Table IV give, respectively, the mean values of dΔ[CaT]/dt (including contributions from dΔ[Cafura-2]/dt) that were obtained with 0.5–2 and 2–4 mM fura-2. The values in parentheses represent the ratio of the corresponding value in column 4 or 5 to that in column 3. According to all three methods of calculation, the peak value of dΔ[CaT]/dt was increased when the resting concentration of fura-2 was increased from 0 to 0.5–2 mM (cf. columns 3 and 4). The value was then decreased when resting [fura-2] was increased to 2–4 mM (column 5). With model 1 and model 2:0.25Δ[Ca], the value with 2–4 mM fura-2 was slightly greater than that with 0 mM fura-2, whereas with model 2:Δ[Ca] the value was slightly less.

Estimate of the Association Rate Constant for Ca and Myoplasmic Fura-2

As described above in connection with Fig. 3 and Table II, Baylor and Hollingworth (1988) and Klein et al. (1988) used the antipyrylazo III–related Δ[Ca] signal to estimate the value of the rate constant k1 for the reaction between Ca and fura-2 inside a muscle fiber, Eq. 10 or 11. The value of k1 can be similarly estimated in our

| Fiber | Method                  | 0 mM  | 0.5–2.0 mM | 2.0–4.0 mM |
|-------|-------------------------|-------|------------|------------|
|       | dΔ[CaT]/dt (μM/ms)      |       |            |            |
| 323921| Model 1                 | 128   | 149 (1.16) | 132 (1.03) |
|       | Model 2:Δ[Ca]           | 194   | 217 (1.12) | 169 (0.87) |
|       | Model 2:0.25Δ[Ca]       | 125   | 164 (1.31) | 148 (1.18) |
| 616921| Model 1                 | 169   | 208 (1.23) | 190 (1.12) |
|       | Model 2:Δ[Ca]           | 239   | 257 (1.08) | 222 (0.93) |
|       | Model 2:0.25Δ[Ca]       | 152   | 228 (1.50) | 195 (1.28) |
| 618922| Model 1                 | 116   | 140 (1.21) | 119 (1.03) |
|       | Model 2:Δ[Ca]           | 177   | 192 (1.08) | 173 (0.98) |
|       | Model 2:0.25Δ[Ca]       | 115   | 144 (1.25) | 134 (1.17) |
| Mean  | Model 1                 | 138   | 166 (1.20) | 147 (1.06) |
|       | Model 2:Δ[Ca]           | 203   | 222 (1.09) | 188 (0.93) |
|       | Model 2:0.25Δ[Ca]       | 131   | 179 (1.35) | 159 (1.21) |

Column 1 gives the fiber reference with, in parentheses, the values of [Cafura-2] and [fura-2] in millimolar in the end pool solutions, separated by a slash mark. Column 2 gives the method used to estimate dΔ[CaT]/dt, the rate of SR Ca release, from myoplasmic free [Ca] (Table I). Columns 3–5 give peak values of dΔ[CaT]/dt (including any contributions from dΔ[Cafura-2]/dt) obtained with 2.1–3.1 mM PDAA and 0, 0.5–2.0, or 2.0–4.0 mM fura-2 at the optical site, as indicated. The numbers in parentheses in columns 4 and 5 represent the values in these columns divided by the value in column 3. Additional information is given in Table III.
fura-2 experiments from the PDA A Δ[Ca] signal. Fig. 10 B shows an example of simultaneously recorded Δ[Ca] and Δ[Cafura-2] signals in a fiber that contained 2.524 mM PDA A and 0.504 mM fura-2. In the first few milliseconds after the action potential, the Δ[Cafura-2] signal rapidly increased from zero to a new level, driven by the transient Δ[Ca] signal.

To apply Eq. 10 to the Δ[Ca] and Δ[Cafura-2] signals, it is convenient to represent the concentration terms in the equation by [Cafura-2] = [Cafura-2]0 + Δ[Cafura-2], [fura-2] = [fura-2]0 + Δ[fura-2], and [Ca] = [Ca]0 + Δ[Ca]. The subscript zero denotes resting concentration and Δ denotes a change with respect to this concentration. Once these substitutions are made into Eq. 10, use is made of the equilibrium relation 

\[ k_1[Ca]_0[fura-2]_0 - k_{-1}[Cafura-2]_0 = 0 \]

Since the dissociation of Ca from Cafura-2 inside myoplasm appears to be slow, with \( k_{-1} = 12-17 \text{ s}^{-1} \) at 6-16°C (column 5 in Table II), the term that contains \( k_{-1} \) can be neglected during the period of the rapid increase in Cafura-2 concentration in Fig. 10 B. Since \( Δ[Ca][fura-2] \gg Δ[Cafura-2][Ca]_0 \) during this time, it is easy to show that the resulting equation can be written:

\[
d[Δ[Cafura-2]]/dt = k_1Δ[Ca][fura-2] \tag{15}
\]

Integration from 0 to \( t \) gives

\[
k_1 = \frac{1}{Δ[Ca]_t} \ln \left( \frac{[fura-2]_0}{[fura-2]_0 - Δ[Cafura-2]} \right) \tag{16}
\]

Δ[Cafura-2] denotes the value at time = \( t \). In practice, the integration was carried out from the time of the stimulus to the time that Δ[Cafura-2] had reached 80-90% of its final value.

This procedure gave \( k_1 = 0.50 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \) for the traces in Fig. 10 B (resting [PDA A] = 2.524 mM and resting [fura-2] = 0.504 mM). Two other sets of traces from the same fiber gave 0.61 \times 10^8 \text{ M}^{-1}\text{s}^{-1} with resting [fura-2] = 1.146 mM and 0.87 \times 10^8 \text{ M}^{-1}\text{s}^{-1} with resting [fura-2] = 2.344 mM (Fig. 10 C). At larger resting concentrations of fura-2, the estimates of Δ[Ca] were unreliable. At resting [fura-2] \approx 4 \text{ mM}, the PDA A Δ[Ca] signal was nearly undetectable and its amplitude was sufficiently small that the value of \( k_1 \) must have been \( ≥ 1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \).

The results in the preceding paragraph suggest that the apparent value of \( k_1 \) progressively increases as resting [fura-2] is increased from 0.5 to 4 mM. Values of \( k_1 \) estimated in two other fibers showed a similar trend. For all three fibers, the mean values of \( k_1 \) (interpolated from the measurements) were 0.35 \times 10^8 \text{ M}^{-1}\text{s}^{-1} with resting [fura-2] = 0.5 mM, 0.43 \times 10^8 \text{ M}^{-1}\text{s}^{-1} with resting [fura-2] = 1 mM, and 0.69 \times 10^8 \text{ M}^{-1}\text{s}^{-1} with resting [fura-2] = 2 mM; the PDA A concentration was 2.5-3.1 mM. The mean value obtained with 0.5-1 mM fura-2, 0.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}, is similar to the value obtained by Klein et al. (1988) with 0.05 mM fura-2 after correction for the expected underestimate in the amplitude of Δ[Ca] by antipyrylazo III (value marked with an asterisk in column 4 in Table II). It is also similar to the value obtained by Baylor and Hollingworth (1988) with 0.3-0.8 mM fura-2, as reported by Hollingworth et al. (1992) (column 4 in Table II).
This article describes two effects of fura-2 on SR Ca release elicited by action potentials in cut fibers from *Rana temporaria*. The first is an increase in Δ[Ca\(_{\text{f}}\)] (the amount of Ca released from the SR) and dΔ[Ca\(_{\text{f}}\)]/dt (the rate of Ca release) as the concentration of resting fura-2 at the optical site was increased from 0 to 0.5–2 mM. On average, dΔ[Ca\(_{\text{f}}\)]/dt increased from 138 to 166 µM/ms (Table IV, model 1). This increase is qualitatively similar to that obtained in intact fibers, also from *Rana temporaria*, which were injected with fura-2 and analyzed for SR Ca release in a similar manner (Baylor and Hollingworth, 1988; Hollingworth et al., 1992). These authors attributed the increase to a reduction of Ca inactivation of Ca release that resulted from fura-2's ability to reduce the myoplasmic free [Ca] transient. Their change in dΔ[Ca\(_{\text{f}}\)]/dt, however, was larger than ours. For example, with 0 mM fura-2, their value of dΔ[Ca\(_{\text{f}}\)]/dt was 75–128 µM/ms (mean value, 98 µM/ms), and with 2–3 mM fura-2, values as large as 200 µM/ms were obtained (mean value, 179 µM/ms). Thus, the reduction of Ca inactivation of Ca release by fura-2 appears to be more pronounced in intact fibers than in cut fibers. A comparison of these numbers suggests that this effect could be due to the presence of a greater degree of Ca inactivation of Ca release in intact fibers in the absence of fura-2.

The second effect of fura-2 was a decrease in SR Ca release that was observed at resting concentrations >2–3 mM. Concentrations of several millimolar fura-2 are easily achieved and maintained in our experiments by diffusion of fura-2 from the end pools into the optical site. The value of dΔ[Ca\(_{\text{f}}\)]/dt decreased by about half when the concentration of resting fura-2 was increased from 2–4 to 5–6 mM. Hollingworth et al. (1992) also observed a decrease with concentrations of fura-2 >3 mM in two heavily injected fibers; this decrease, however, was also accompanied by a slowing of the waveform of dΔ[Ca\(_{\text{f}}\)]/dt, an effect not detected in our experiments. Hollingworth et al. (1992) tentatively attributed these effects to injection damage, an explanation that does not apply to our results.

A possible explanation for the decrease in dΔ[Ca\(_{\text{f}}\)]/dt that we observed with concentrations of fura-2 >2–3 mM is that fura-2 reduces myoplasmic free [Ca] and that this, in turn, reduces Ca-induced Ca release (Jacquemond et al., 1991). If so, the reduction of Δ[Ca] is too small to be detected with PDAA.

Another possibility is that a large concentration of fura-2 decreases SR Ca release by some means other than by buffering Ca, perhaps by an increase in ionic strength (fura-2 is pentavalent at neutral pH) or by a pharmacological action. Along these lines, a clue may be provided by the experiment shown in Fig. 7. Between 4 and 6 mM, the relation between SR Ca content and the resting concentration of fura-2 is approximately constant and does not show any hysteresis (Fig. 7 C). Consequently, if the effect of resting fura-2 on SR Ca release were due to Ca complexation, which is a rapid process, the relation between either the amount of SR Ca release or the peak rate of release and resting [fura-2] would not be expected to show hysteresis. Both Fig. 7 A and Fig. 7 B, however, show hysteresis between 4 and 6 mM resting fura-2. This raises the possibility that a change in the concentration of resting fura-2 in the range of 4–6 mM does not affect SR Ca release immediately but requires several...
minutes to develop, a delay that is difficult to reconcile with the rapid action expected for rapid Ca buffering.

Additional information about the effects of fura-2 on SR Ca release is given in the following article (Jong et al., 1993).

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