Calcium Signals Recorded from Cut Frog Twitch Fibers Containing Tetramethylmurexide

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ABSTRACT   The Ca indicator tetramethylmurexide was introduced into cut fibers, mounted in a double-Vaseline-gap chamber, by diffusion from the end-pool solutions. The indicator diffused rapidly to the central region of a fiber where optical recording was done and, if removed, diffused away equally fast. The time course of concentration suggests that, on average, a fraction 0.27 of indicator was reversibly bound to myoplasmic constituents and the free diffusion constant was 1.75×10^{-6} cm^2/s at 18°C. The shape of the resting absorbance spectrum suggests that a fraction 0.11–0.15 of tetramethylmurexide inside a fiber was complexed with Ca. After action potential stimulation, there was a rapid transient change in indicator absorbance followed by a maintained change of opposite sign. The wavelength dependence of both changes matched a cuvette Ca-difference spectrum. The amplitude of the early peak varied linearly with indicator concentration and corresponded to an average rise in free [Ca] of 17 μM. These rather diverse findings can be explained if the sarcoplasmic reticulum membranes are permeable to Ca-free indicator. Both Ca-free and Ca-complexed indicator inside the sarcoplasmic reticulum would appear to be bound by diffusion analysis and the Ca-complexed form would be detected by the resting absorbance spectrum. The transient change in indicator absorbance would be produced by myoplasmic Ca reacting with indicator molecules that freely diffuse in myoplasmic solution. The maintained signal, which reports Ca dissociating from indicator complexed at rest, would come from changes within the sarcoplasmic reticulum. A method, based on these ideas, is described for separating the two components of the tetramethylmurexide signal. The estimated myoplasmic free [Ca] transient has an average peak value of 26 μM at 18°C. Its time course is similar to, but possibly faster than, that recorded with antipyrylazo III (Maylie, J., M. Irving, N. L. Sizto, and W. K. Chandler. 1987. *Journal of General Physiology*. 89:83–143).

INTRODUCTION

The preceding article (Maylie et al., 1987*b*) describes optical signals recorded from cut muscle fibers containing the Ca indicator antipyrylazo III. After addition to the end pools, antipyrylazo III slowly appears at the optical recording site at the center of the fiber. An analysis of the time course of the indicator concentration indicates that approximately two-thirds of the indicator is bound to or sequestered by myoplasmic constituents. If this immobilized indicator can react normally with Ca, the free [Ca] transient associated with an action potential has a peak value of ~3 μM. On the other hand, if only freely diffusible indicator reacts with Ca, the peak is considerably higher, ~40 μM. It seems likely that the actual amplitude of the myoplasmic free [Ca] transient lies somewhere between these extreme values.

The function of the [Ca] transient is to provide Ca to complex the Ca-regulatory sites on troponin so that contraction can occur. If free [Ca] reaches only 3 μM, the peak fractional occupancy of the sites is calculated to be slightly less than half; if [Ca] reaches 40 μM, peak occupancy exceeds 0.9 (Maylie et al., 1987*b*). It is clearly of interest to know where in the range 3–40 μM the amplitude of the [Ca] transient lies. To help resolve this uncertainty, we decided to use another class of Ca indicators, murexide and tetramethylmurexide, which have chemical properties different from those of antipyrylazo III and arsenazo III.

Murexide plays a prominent role in the history of Ca measurement in muscle. It was the indicator first used by Jobsis and O'Connor (1966) to report an increase in myoplasmic Ca after excitation. Unfortunately, the magnitude of free [Ca] cannot be determined from their results and the indicator has been little used since. Our preliminary experiments gave similar results with both murexide and tetramethylmurexide, so that one of these, tetramethylmurexide, was selected for the work described in this article. One advantage of tetramethylmurexide over murexide is its reduced sensitivity to pH (Gysling and Schwarzenbach, 1949; Ohnishi, 1978).

This article describes our tetramethylmurexide results. When the indicator is added to the end-pool solutions, it rapidly diffuses to the center of a cut fiber, where the optical measurements are made. If removed, it diffuses away equally fast. On average, 27% of the indicator is estimated to be bound or sequestered inside fibers compared with 68% for antipyrylazo III, an encouraging finding.

The resting absorbance spectrum, however, suggests that 11–15% of tetramethylmurexide inside muscle is complexed with Ca. Since the dissociation constant of the indicator for Ca is large, 2–3 mM, and the resting myoplasmic free [Ca] is small, no greater than 0.1–0.2 μM (Blinks et al., 1982), <0.01% of myoplasmic tetramethylmurexide should be complexed with Ca. The resting spectrum, then, shows that some of the indicator inside muscle is not free in myoplasmic solution; it either must be complexed with Ca, perhaps by being inside a high [Ca] compartment, or for some other reason must have a Ca-like absorbance spectrum.

After action potential stimulation, tetramethylmurexide undergoes an early change in absorbance. This has the wavelength dependence expected for an increase in Ca. Unexpectedly, after this early signal decays, there is a maintained

absorbance change of opposite sign. The wavelength dependence of this late signal also matches the Ca-difference spectrum. This signal, corresponding to a decrease in Ca, must come from tetramethylmurexide molecules that change their absorbance spectrum from the Ca-complexed shape in resting muscle to the Ca-free shape after stimulation. It is the early transient change in absorbance that is likely to arise from tetramethylmurexide in myoplasmic solution, and this change corresponds to a value of 20–30 μM for peak free [Ca]. We believe that this represents the best available estimate of the amplitude of the free [Ca] transient in frog twitch fibers at 18°C.

A preliminary report of some of the work has been given (Maylie et al., 1985).

METHODS

The experimental methods and analysis are similar to those used in the three preceding articles (Irving et al., 1987; Maylie et al., 1987a, b). Cut fibers were mounted in a double-Vaseline-gap chamber. The end-pool regions were exposed for 2 min to a 0.01% saponin solution to make the end segments permeable to the Ca indicator tetramethylmurexide (Calbiochem-Behring Corp., La Jolla, CA). The indicator was initially dissolved in distilled water at twice the desired concentration. Just before each experiment, the final end-pool solution was mixed from equal volumes of this indicator solution and a two-times concentrate of internal solution. The mixing was done at the last minute since a dark precipitate frequently appeared after several hours. The concentration of tetramethylmurexide was measured optically either in the final solution, just before exchanging with the end pools, or, later in the experiment, in the end-pool solution itself. A sample of solution was placed in a cuvette with a 100-μm path length and the absorbance at 520 nm was measured with a spectrophotometer (model Lambda 3 or Lambda 3B, Perkin-Elmer Corp., Norwalk, CT). In all experiments, the central pool contained Ringer's solution at ~18°C and the membrane potential was held at −90 mV.

Calibrations were carried out using a Perkin-Elmer spectrophotometer. The routine calibration solution contained 150 mM KCl, 2 mM MgCl$_2$, 10 mM PIPES, 0.025–0.5 mM tetramethylmurexide, 0–10 mM CaCl$_2$, and pH adjusted to 7.0. The exact level of Mg and pH is unimportant (Gysling and Schwarzenbach, 1949; Ohnishi, 1978; Ogawa et al., 1980). With 0.025 mM indicator at 20°C, the Ca-free resting absorbance spectra were closely similar for $0 \leq$ [Mg] ≤ 5 mM and $6.5 \leq$ pH ≤ 7.5; the changes in absorbance produced by adding Ca were also similar, as were the apparent dissociation constants. Measurements of absorbance at different concentrations of Ca and indicator were analyzed on the assumption that the stoichiometry of the Ca:indicator complex was 1:1 (Ohnishi, 1978). Concentrations of total Ca and total indicator were corrected for Ca:indicator complexation to give concentrations of free Ca and free indicator. The analysis provided the wavelength dependence of the molar extinction coefficients for Ca-free indicator and Ca-complexed indicator as well as the apparent dissociation constant for Ca.

The following values, obtained from the calibrations, were used in the analysis of absorbance changes measured in muscle fibers: (a) 1.58 × 10^4 M^{-1} cm^{-1} for ϵ(520), the molar extinction coefficient for Ca-free indicator at 520 nm; 520 nm is near the isosbestic wavelength, which in our measurements was 519 nm; (b) −0.91 × 10^4 M^{-1} cm^{-1} for $\Delta\epsilon$(570), the change in molar extinction coefficient at 570 nm when Ca is complexed; (c) 2.6 mM for K_D, the apparent dissociation constant of indicator for Ca, measured with 0.5 mM indicator at 15°C. These values are in good agreement with those of Ohnishi (1978) and Ogawa et al. (1980), who made measurements under similar but not identical conditions. In our calibrations, K_D decreased 10–12% when indicator concentration was

increased from 0.025 to 0.5 mM. This relatively small change is consistent with a predominant 1:1 stoichiometry for Ca:indicator in this concentration range (Ohnishi, 1978). We have no evidence that suggests a different stoichiometry at the higher indicator concentrations, up to 3 mM, used in our muscle experiments.

The above values of molar extinction coefficients have been used for muscle absorbance measurements made with 10-nm bandpass filters. Active 570-nm signals were routinely measured with a 30-nm bandpass filter, in which case a value of -0.84×10^4 M^{-1} cm^{-1} was used for $\Delta\epsilon(570)$; this value was determined from $\Delta\epsilon(570) = -0.91 \times 10^4$ M^{-1} cm^{-1} for a 10-nm bandpass filter and the factor 1.08 obtained by scaling the indicator-related 570-nm trace taken with a 30-nm bandpass filter to fit that taken simultaneously with a 10-nm bandpass filter.

[TMX]$_T$, the total concentration of indicator at the site of optical recording, was estimated from indicator-related absorbance measured at 520 nm. This estimate used Beer's law (Eq. 3 in Maylie et al., 1987a), the value for $\epsilon(520)$ given above, and an optical path length for the myoplasmic space equal to 0.7 times the fiber diameter (Baylor et al., 1983).

Changes in indicator-related absorbance at 570 nm were used to estimate changes in the concentration of the Ca:tetramethylmurexide complex, [CaTMX], again using Beer's law, the above values for $\Delta\epsilon(570)$, and 0.7 times the fiber diameter for the optical path length. For a stoichiometry of 1:1 (Ohnishi, 1978), free [Ca] can be calculated from the relation

$$\frac{[Ca] \cdot ([TMX]_T - [CaTMX])}{[CaTMX]} = K_D. \tag{1}$$

This equation assumes that all the tetramethylmurexide is available to react with Ca and that the reaction is essentially instantaneous. Since myoplasmic [Ca] is much less than the K_D, the speed of reaction is given by the rate constant for dissociation of the Ca:indicator complex. For murexide, this is $\geq 1.6 \times 10^5$ s^{-1} at 10°C (Geier, 1968). Tetramethylmurexide should react with similar speed (Ogawa et al., 1980), so that the reaction delay would be no greater than $1/(1.6 \times 10^5)$ s = 6 μs, which is effectively instantaneous in our muscle experiments.

In the measurements described in this article, 570- and 690-nm filters with a 30-nm bandpass were used, respectively, in the λ_2 and λ_3 positions (Fig. 2 in Irving et al., 1987). Filters with a 10-nm bandpass were usually used in the λ_1 position, the standard filter being 520 nm; occasionally, however, a 480-nm filter with a 30-nm bandpass was used. Resting and active absorbance measurements were corrected for the intrinsic contribution, estimated from the signal at 690 nm (a wavelength not absorbed by tetramethylmurexide) and the wavelength dependence given by Eqs. 1 and 2 in Maylie et al. (1987a). All active signals were recorded using 0.625-kHz eight-pole Bessel filters. The 690-nm signals were additionally filtered by a 0.1-kHz Gaussian digital filter (Colquhoun and Sigworth, 1983) to further reduce noise introduced by the intrinsic correction.

All measurements of $A(\lambda)$ and $\Delta A(\lambda)$ in this article have had their intrinsic contributions subtracted, except for the traces in Fig. 3A.

RESULTS

Experiments were carried out on eight fibers in Ringer's solution in which Ca signals were elicited by action potential stimulation. Information about these fibers and their viability during the experiments is given in Table I.

Diffusion of Tetramethylmurexide in Cut Muscle Fibers

Fig. 1A shows the tetramethylmurexide concentration at the site of optical recording plotted against time after addition to the end pools. The concentration at the optical site eventually exceeded the end-pool level, 1.79 mM, a concentrating effect similar to but less marked than that found with arsenazo III and antipyrylazo III (Maylie et al., 1987a, b). The continuous curve shows a least-squares fit of Eqs. 6 and 8 in Maylie et al. (1987a), derived from free diffusion plus linear reversible binding.

Fig. 1B shows results from another experiment in which 1.60 mM indicator was present in the end-pool solutions for 59 min. After removal, the indicator concentration at the optical site decreased and finally became indistinguishable from zero. Thus, tetramethylmurexide, unlike arsenazo III and antipyrylazo III

TABLE I

Change in Electrical Parameters during the Time Course of Experiments on Cut Muscle Fibers Containing Tetramethylmurexide

(1) Fiber reference	(2) Duration of exper- iment	(3) Temper- ature	(4) Diameter	(5) Action potential	(6) Holding current	(7) r_m	(8) $r_e/(r_e + r_i)$
	min	*°C*	*μm*	*mV*	*−nA*	*MΩ·cm*	
061584.2	101	17.4–17.5	117–101	131–129	101–105	0.050–0.033	0.967–0.961
061984.1	229	18.6–18.7	98–94	133–127	54–49	0.069–0.054	0.981–0.976
062684.2	56	19.0–17.3	94–98	127–125	129–128	0.058–0.054	0.945–0.950
062784.1	113	17.1–16.4	98–101	129–128	76–77	0.085–0.067	0.966–0.966
071184.1	133	18.8–18.9	70–67	133–125	40–43	0.097–0.054	0.967–0.967
101784.2	82	17.3–17.9	102–108	141–132	55–51	0.072–0.047	0.982–0.983
101884.1	92	18.6–17.6	71–71	134–131	25–25	0.165–0.124	0.973–0.975
101984.1	112	18.6–18.1	104–90	138–134	31–31	0.085–0.035	0.985–0.984

Column 1 gives fiber reference. Column 2 gives the duration of the experiment beginning from the time of saponin treatment. Columns 3–8 give values of temperature, fiber diameter, action potential amplitude, holding current (sign reversed), r_m, and $r_e/(r_e + r_i)$. The first value in each column was obtained ~20 min after saponin treatment; the second value was obtained at the end of the experiment. Sarcomere spacing, 3.7–3.9 μm; holding potential, −90 mV.

(Maylie et al., 1987a, b), can completely leave the optical site, presumably by diffusing along the fiber into the end pools. The entire time course of concentration is well fitted by the continuous curve, calculated from the equations for diffusion plus linear reversible binding using a single set of parameters.

Values of the fitted parameters $D/(R + 1)$ and $(R + 1)$ are given in columns 4 and 5 of Table II; $D/(R + 1)$ is the apparent diffusion constant of indicator, R is the ratio of bound to free indicator, and D is the diffusion constant of free indicator. The average value of $(R + 1)$ was 1.371, which indicates that 0.371/1.371 = 0.27 of the total tetramethylmurexide inside a fiber appears to be bound or sequestered. This fraction, though appreciable, is considerably smaller than the fractions 0.73 and 0.68 obtained for arsenazo III and antipyrylazo III, respectively (Table II, Maylie et al., 1987a; Table IIA, Maylie et al., 1987b).

The average value of D for tetramethylmurexide was 1.75×10^{-6} cm^2/s at 17.9°C (Table II, column 6). This value should be compared with the results of Kushmerick and Podolsky (1969). They studied the diffusion of compounds of various molecular weights in skinned muscle fibers and found that D for sucrose,
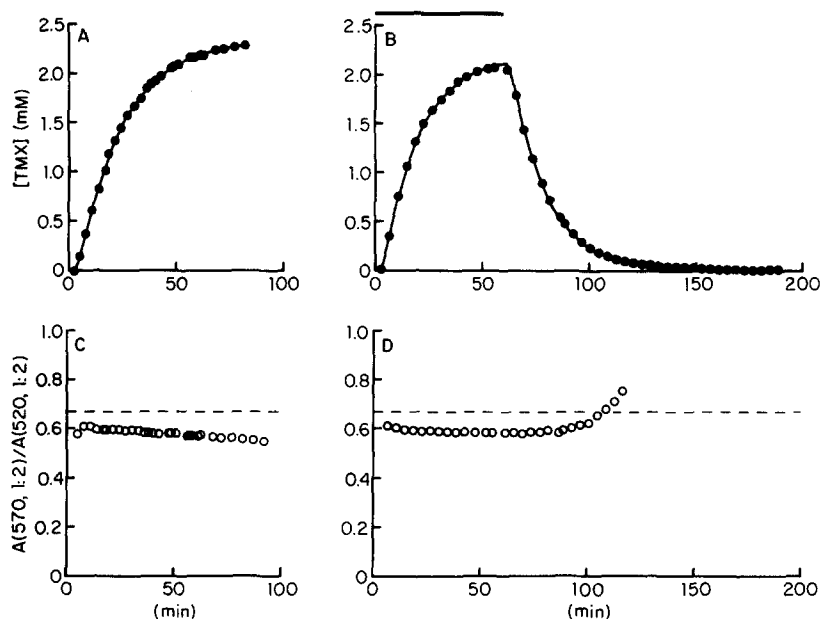

FIGURE 1. Diffusion of tetramethylmurexide in cut muscle fibers. (*A*) Tetramethylmurexide concentration at the optical recording site plotted as a function of time after its addition to the end-pool solutions 51 min after saponin treatment. The theoretical curve shows a least-squares fit of Eqs. 6 and 8 of Maylie et al. (1987*a*), derived from diffusion plus linear reversible binding. The parameters used for the curve are given in Table II. Fiber 071184.1; sarcomere spacing, 3.9 μm; end-pool concentration of indicator, 1.79 mM. (*B*) Similar to *A*, except that indicator was added to the end pools 40 min after saponin treatment and was removed 59 min later; the duration of tetramethylmurexide exposure is indicated by the horizontal bar. Fiber 061984.1; sarcomere spacing, 3.9 μm; indicator concentration, 1.60 mM. (*C* and *D*) $A(570, 1{:}2)/A(520, 1{:}2)$ plotted against time, from the experiments in *A* and *B*, respectively. The measurements of $A(570)$, made with a 30-nm bandpass filter in the λ_2 position, were scaled to correspond to measurements with a 10-nm bandpass filter. The dashed lines show the value measured in a calibration solution that contained no Ca. Measurements of resting absorbance in this and subsequent figures have been corrected for intrinsic contributions.

which has nearly the same molecular weight as tetramethylmurexide, was 2.1×10^{-6} cm^2/s at 20°C. Considering the difference in temperature, these values for tetramethylmurexide in cut fibers and sucrose in skinned fibers are in good agreement.

Resting Spectrum of Tetramethylmurexide

Fig. 2 shows the wavelength dependence of tetramethylmurexide absorbance in a resting fiber. The data have been normalized by the value at the isosbestic wavelength, 520 nm. The dashed curve labeled "$f = 0$" shows the absorbance spectrum of Ca-free indicator measured in a cuvette. The other dashed curve, labeled "$f = 0.3$," shows the spectrum of indicator with a fraction 0.3 complexed with Ca and 0.7 Ca-free. The data from the fiber (circles) lie between the two dashed curves. A least-squares fitting routine was used to estimate the fraction of tetramethylmurexide that appears to be complexed with Ca, $f = 0.15$ in this experiment, and the continuous curve shows the spectrum calculated from cuvette spectra using this value of f.

TABLE II

Parameters Associated with the Analysis of Tetramethylmurexide Diffusion in Cut Fibers, Including Reversible Binding

(1) Fiber reference	(2) Indicator concentration	(3) Indicator exposure	(4) $D/(R + 1)$	(5) $(R + 1)$	(6) D
	mM	min	$\times\ 10^{-6}\ cm^2/s$		$\times\ 10^{-6}\ cm^2/s$
061584.2	1.76	42	1.274	1.412	1.799
061984.1 (1 *B*)	1.60	59	1.224	1.368	1.674
062684.2	1.12	35	1.380	1.267	1.748
062784.1	1.75	31	1.342	1.369	1.837
071184.1 (1 *A*)	1.79	82	1.003	1.309	1.313
101784.2	1.34	57	1.517	1.503	2.281
101884.1	1.39	65	1.167	1.371	1.600
Mean			1.272	1.371	1.750
SEM			0.062	0.028	0.110

Column 1 gives fiber reference; the numbers and letters in parentheses refer to figures in which theoretical diffusion curves are shown. Column 2 gives the concentration of indicator used in the end-pool solution, determined from $A(520)$ measured at the time of the experiment (see Methods). Column 3 gives the period of time that indicator was present. Columns 4 and 5 give parameters associated with fitting Eqs. 6 and 8 in Maylie et al. (1987*a*) to the experimental points. Column 6 gives the product of columns 4 and 5. Fiber 101984.1 is not included since the indicator concentration was not measured in the end-pool solution. The indicator was added to the end pools 19–51 min (average value, 33 min) after saponin treatment. Average temperature, 17.9°C. Other information is given in Table I.

Estimates of resting myoplasmic free [Ca] vary according to method but are never greater than 0.1–0.2 μM (Blinks et al., 1982). Since the K_D of tetramethylmurexide for Ca is 2–3 mM, <0.01% of indicator inside myoplasm should be complexed with Ca. The absorbance spectrum shown in Fig. 2, then, suggests that some of the indicator inside the fiber is not dissolved in myoplasm; it appears to be either located inside a high [Ca] environment, such as the sarcoplasmic reticulum, or complexed to internal constituents in a manner that produces a Ca-like absorbance spectrum. Whatever the explanation, the tetramethylmurexide in question is either inside the fiber or its transverse tubular system and is not in the external solution; light intensities measured without the fiber in the

optical path were the same as those measured at the beginning of the experiment before indicator was introduced into the end pools. The transverse tubular system seems an unlikely candidate for the high [Ca] compartment since it occupies only 0.3% of the fiber volume (Peachey, 1965; Mobley and Eisenberg, 1975).

Throughout this article, measurements of tetramethylmurexide absorbance in fibers will be compared with cuvette calibrations of Ca-containing and Ca-free solutions. If the wavelength dependence can be matched by such calibrations, as in Fig. 2, the muscle data will be described in terms of Ca complexation. Although this convention facilitates description of the results, one should bear in mind that
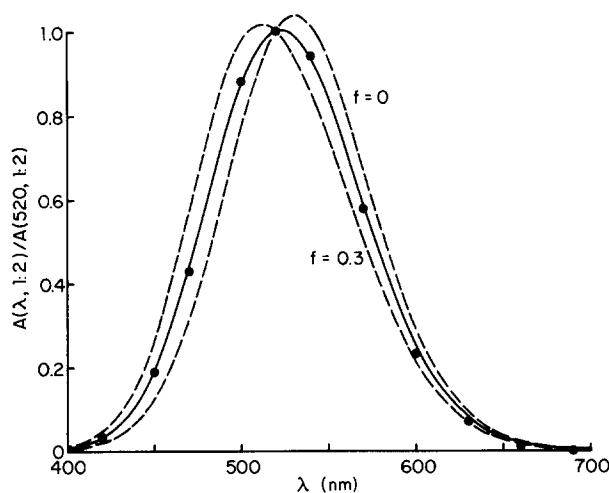

FIGURE 2.   Wavelength dependence of tetramethylmurexide absorbance in a resting muscle fiber. The circles show $A(\lambda, 1{:}2)/A(520, 1{:}2)$ plotted against wavelength. The continuous calibration curve shows the absorbance spectrum for indicator that is 0.15 fractionally complexed with Ca; the amount of complexation was determined by a least-squares computer program. The dashed curves show 0 and 0.3 fractional complexation. Same fiber as in Fig. 1A. The measurements were made 34–48 min after adding indicator to the end pools or 85–99 min after saponin treatment; the concentration at the optical site was 1.75–2.06 mM.

some of the effects attributed to Ca:indicator complexation might be due to some other interaction of the indicator that spectrally mimics that with Ca.

Resting $A(570)/A(520)$ is a convenient monitor of the fractional amount of tetramethylmurexide complexed by Ca. Panels C and D of Fig. 1 show measurements of this ratio for the experiments in panels A and B, respectively; the dashed lines indicate the value for Ca-free indicator. As soon as reliable measurements of indicator-related $A(570)/A(520)$ could be made, 5–10 min after addition of tetramethylmurexide to the end pools, the ratio was well below the [Ca] = 0 level. As time progressed, $A(570)/A(520)$ usually decreased slightly. This was more marked in the experiment in Fig. 1C than in that in Fig. 1D. The last points in Fig. 1D, obtained with little indicator inside the fiber, are unreliable

owing to the relatively large intrinsic correction. Although the cause of the gradual change is not known, it seems clear that the predominant process that underlies Ca complexation, such as tetramethylmurexide entering a high [Ca] space, happens within 10 min.

The resting absorbance spectrum in Fig. 2, best fitted with $f = 0.15$, was measured 34–48 min after adding tetramethylmurexide to the end pools. At the time of the measurements, the concentration of indicator was 1.75–2.06 mM at the optical site. Complete resting spectra were measured in two other fibers, giving $f = 0.13$ (36–39 min after indicator addition, 2.17–2.23 mM indicator during the measurements) and $f = 0.11$ (43–54 min, 1.98–2.06 mM). The average from the three fibers, $f = 0.13$, will be used throughout this article for the resting fraction of indicator that appears to be complexed with Ca.

Resting Dichroism in Cut Fibers Containing Tetramethylmurexide

Resting dichroism at 520 nm increased with indicator concentration, whereas that at 690 nm, a wavelength at which tetramethylmurexide is transparent, remained constant. Indicator-related dichroism, $A(520, \delta) - A(690, \delta)$, was positive and approximately proportional to indicator-related $A(520, 1:2)$, or tetramethylmurexide concentration. In five fibers, the ratio [tetramethylmurexide-related dichroism]:[tetramethylmurexide-related $A(520, 1:2)$] varied from 0.021 to 0.041 and had an average value of 0.029 (0.003 SEM). This value is significantly different from zero, $P < 0.005$ using the two-tailed t test. It is also significantly different, with $0.025 < P < 0.05$ using the two-tailed t test, from the corresponding values 0.043 and 0.042 obtained, respectively, with arsenazo III and antipyrylazo III (Maylie et al., 1987a, b). The wavelength dependence of the small dichroic component could not be established with certainty owing to scatter in the data; a cuvette spectrum for 0–20% Ca complexation would give a reasonable fit, whereas one based on 100% Ca complexation would not. This small resting dichroism is probably due to indicator molecules bound to oriented structures inside the fiber.

Ca Signals in Cut Fibers That Contain Tetramethylmurexide

In Fig. 3A, the top trace shows an action potential and the next four traces show changes in 1:2 absorbance, uncorrected for the intrinsic signal. The 480-, 570-, and 690-nm traces were from one run and the 520-nm trace was from the next run. In this and other experiments, we were unable to detect any indicator-related active dichroic signal. A small signal may be present but unresolvable since the active changes in indicator absorbance were not very large.

The 690- and 520-nm traces in Fig. 3A have similar waveforms. These signals should be predominantly intrinsic since tetramethylmurexide does not absorb at 690 nm, and 520 nm is close to the isosbestic wavelength for Ca. The 520-nm trace is larger than that at 690 nm, as expected from the wavelength dependence of intrinsic signals in fibers without indicator (Irving et al., 1987).

The 480- and 570-nm traces in Fig. 3A show an extra, fast signal but are contaminated by intrinsic contributions, which must be subtracted to obtain the indicator-related components. The intrinsic signal was assumed to have the same

waveform at different wavelengths with an amplitude that varied as λ^{-n} (Eq. 2 in Maylie et al., 1987a). The method used to estimate n was similar to that used for antipyrylazo III (Maylie et al., 1987b). A linear combination of the simultaneously obtained 690- and 570-nm traces was fitted by a least-squares computer program to either the 480- or 520-nm trace. The fits were good (not shown). The value of n was determined from the scaling constants on the assumption
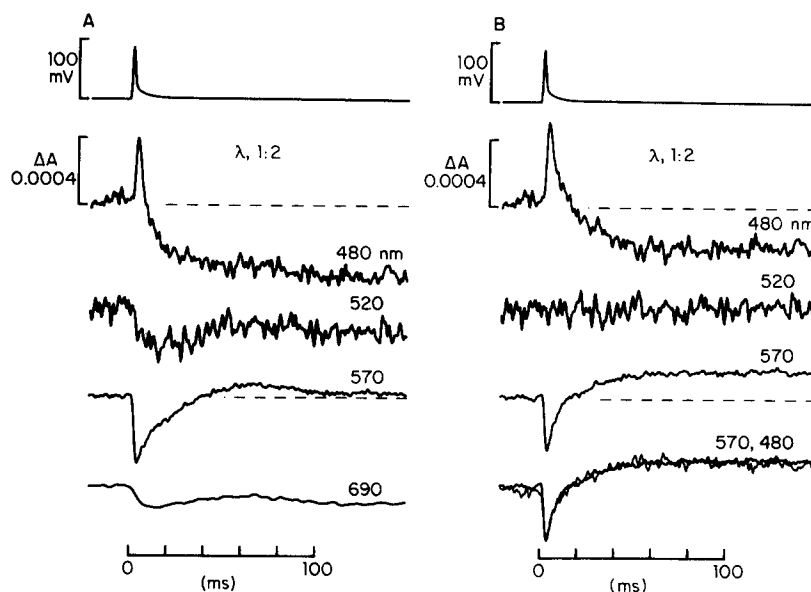

FIGURE 3. Traces of $\Delta A(\lambda, 1{:}2)$ obtained at different wavelengths after action potential stimulation. (A) Action potential and four uncorrected 1:2 absorbance traces with wavelengths indicated. The action potential has been attenuated by a 0.625-kHz eight-pole Bessel filter; its actual amplitude, measured on a storage oscilloscope, was 128 mV. The 480-, 570-, and 690-nm traces were obtained with 30-nm bandpass filters; the noisier 520-nm trace was obtained with a 10-nm bandpass filter. (B) Action potential and three absorbance traces corrected for the intrinsic contribution as described in the text; $n = 1.71$ (480 and 570 nm) and 2.01 (520 nm). The bottom pair of traces shows the 570-nm trace superimposed on the 480-nm trace scaled by −0.625. Same fiber as in Figs. 1A and 2; tetramethylmurexide concentration, 1.51–1.58 mM; time after saponin treatment, 77–78 min. All traces displayed in this and subsequent figures represent single sweeps (i.e., signal averaging was not used). In subsequent figures, changes in absorbance have been corrected for intrinsic contributions.

that the 690-nm trace was purely intrinsic and the other traces were composed of an intrinsic plus one other waveform. The value of n was 1.71 in the run that included 480 nm and 2.01 in the run with 520 nm. In view of the noise in the traces, these values are in good agreement.

The first three optical traces in Fig. 3B show absorbance changes corrected for the intrinsic contribution. The 520-nm trace is nearly flat. The 480- and

570-nm traces show, respectively, a rapid increase and decrease in absorbance. These changes are transient and soon give way to maintained changes of opposite sign. The 570-nm signal is replotted at the bottom with a least-squares scaled 480-nm signal superimposed. The two traces are the same, within experimental error, which is consistent with the idea that the same process(es) underlies both the 480- and 570-nm signals.

If the tetramethylmurexide signals at all wavelengths are due to a single kind of spectral change, for example, that produced by a change in Ca complexation,
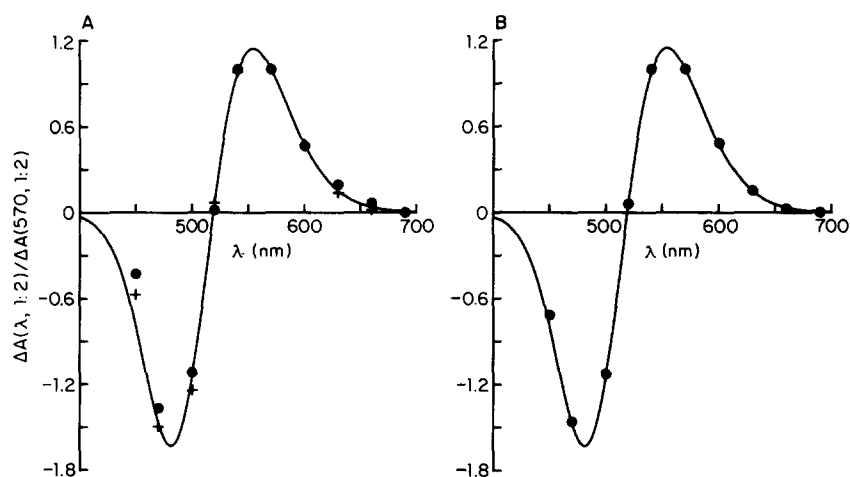

FIGURE 4. Wavelength dependence of the change in tetramethylmurexide absorbance. (A) Peak (●) and late (+, measured at 0.3 s) 1:2 absorbance. Each point has been normalized by the corresponding value obtained from the simultaneously recorded 570-nm trace. The continuous curve is the normalized Ca-difference spectrum determined from cuvette calibrations as described in the Methods. (B) Scaling factors determined by fitting the 1:2 trace at each wavelength by the 570-nm trace taken at the same time; for each trace, the first 150 ms after stimulation was used for the fit. Same Ca-difference spectrum as plotted in A. In both A and B, traces obtained at different wavelengths, using 10-nm bandpass filters, have been compared with simultaneously recorded 570-nm traces obtained with a 30-nm bandpass filter. Before comparison, the 570-nm traces were scaled to correspond to 10-nm bandpass traces. Same fiber as in Figs. 1A, 2, and 3; indicator concentration, 1.75–2.06 mM; time after saponin treatment, 85–99 min.

the early transient and maintained components should have the same wavelength dependence. The circles in Fig. 4A show the peak transient absorbance change normalized by the value at 570 nm and plotted as a function of wavelength. The crosses show the normalized amplitude of the maintained change. The two sets of points are in close agreement and are well fitted by the normalized Ca-difference spectrum (continuous curve). Each entire $\Delta A(\lambda)$ trace was also least-squares fitted by a scaled version of the simultaneously recorded $\Delta A(570)$ trace. Fig. 4B shows the scaling constants, which are in good agreement with the Ca-difference spectrum. Similar results were obtained in two other fibers. The

general conclusion is that the tetramethylmurexide absorbance signal, both the transient and the maintained components, is caused by changes in Ca:indicator complexation; the transient component represents an increase almost certainly caused by the myoplasmic Ca transient, whereas the maintained component represents a decrease caused by dissociation of resting Ca:tetramethylmurexide complexes.

Since Ca:tetramethylmurexide exists as a 1:1 complex in cuvette calibration solutions (Ohnishi, 1978; see pp. 147–148), it was of interest to find out whether the amplitude of the muscle signal varies linearly with indicator concentration.
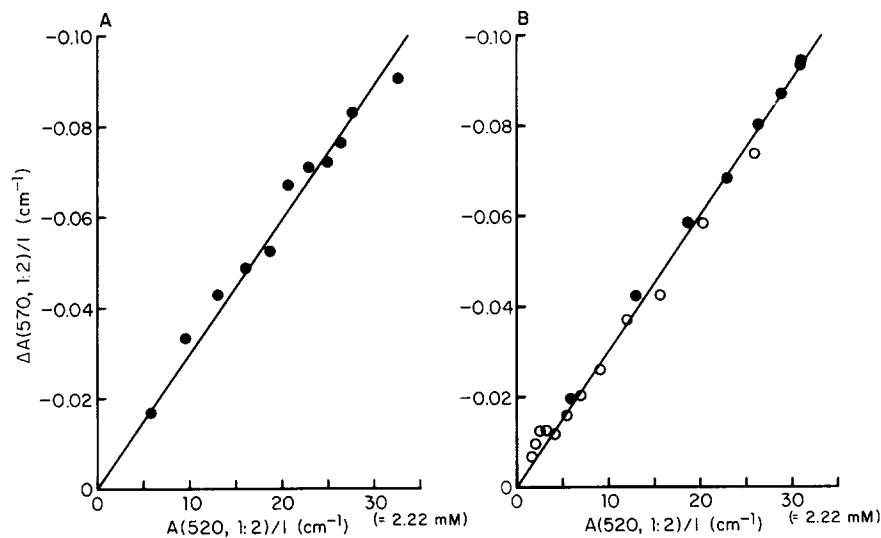

FIGURE 5. Values of peak ΔA(570, 1:2) plotted against A(520, 1:2) or indicator concentration. The A(520) and ΔA(570) points were obtained with 10- and 30-nm bandpass filters, respectively, and have been normalized by optical path length l. (A) From the experiment illustrated in Figs. 1A and 2–4. (B) From a pulsed-exposure experiment. The filled circles were obtained as indicator concentration was increasing and the open circles as it was decreasing. Fiber 062784.1; sarcomere spacing, 3.7 μm. The straight lines represent least-squares fits constrained to intersect the origin; the slope is −0.00297 in A and −0.00301 in B. A value of −0.1 for ΔA(570)/l corresponds to an 11.9-μM increase in the Ca:tetramethylmurexide concentration.

Fig. 5A shows peak transient ΔA(570)/l plotted against A(520)/l, or indicator concentration, from the experiment illustrated in Figs. 1A and 2–4. l is the optical path length and a value of −0.1 for ΔA(570)/l corresponds to Δ[CaTMX] = 11.9 μM. With some scatter, the data follow a linear relation. The line represents a least-squares fit constrained to intersect the origin, with slope −0.00297. Fig. 5B shows similar data from a pulsed-exposure experiment. The filled circles were obtained as indicator concentration was increasing, the open circles as it was decreasing. The linear relation between peak ΔA(570)/l and the indicator concentration was found to be reversible, with slope −0.00301.

The average slope of the relation between $\Delta A(570)/l$ and $A(520)/l$ in four fibers was -0.00296 (0.00018 SEM). This corresponds to a 0.0056 increase in the amount of tetramethylmurexide complexed with Ca. In all experiments, the relation between $\Delta A(570)/l$ and the indicator concentration was linear even at high concentrations, which suggests that the Ca-buffering power of tetramethylmurexide did not change the amplitude of peak free [Ca]. This is not surprising since, owing to the low affinity of this indicator for Ca, the increase in [CaTMX] never exceeded 18 μM.

Peak free myoplasmic [Ca] can be determined if the dissociation constant, K_D, and the fraction of tetramethylmurexide available to react with myoplasmic Ca are known. Two methods have been used to estimate this fraction. The first, based on the shape of the resting absorbance spectrum, assumes that it is the fraction of indicator that is spectrally Ca-free. The second method, based on the diffusion analysis, assumes that it is the fraction of indicator that is freely diffusible ($= 1/[R + 1]$, column 5 of Table II). According to the first method, 0.87 of the indicator would be available, and according to the second method, 0.73 would be available. Consequently, either $0.0056/0.87 = 0.0064$ or $0.0056/0.73 = 0.0077$ of the available tetramethylmurexide would be complexed with Ca, above the resting level, at the peak of the transient. Using $K_D = 2.6$ mM (see p. 147), peak free myoplasmic [Ca] is 16.7 μM with the first method and 20.2 μM with the second method.

The effect of indicator concentration on the ratio of maintained to peak $\Delta A(570)$ was also investigated in the four fibers described in the preceding two paragraphs (not shown). The values were almost independent of concentration, except possibly for a slight decrease in absolute magnitude at high concentrations. The average ratios in three fibers were -0.48, -0.51, and -0.60. The other fiber had a large $\Delta A(690)$ signal, which made the intrinsic correction of the steady level, but not the peak, unreliable. The overall average, -0.53, corresponds to a maintained change of -0.0030 in the fraction of total tetramethylmurexide complexed with Ca.

Ca Signals Associated with Repetitive Stimulation

Fig. 6 A shows three pairs of superimposed traces obtained with 1 and 10 action potential stimulations. The top pair shows the action potentials; the middle pair shows the Ca signals, $\Delta A(570, 1{:}2)$; the bottom pair shows the intrinsic signals, $\Delta A(690, 1{:}2)$.

It is difficult to fully evaluate the effect of repetitive stimulation on the early transient Ca signal because of overlap with the maintained signal. The one effect that seems clear is that the amplitude of the transient component declines during the train. The difference between the peak and steady levels after the 10th action potential was (a) ~0.6 times that after the single action potential and (b) less than the difference between the peak and prestimulus baseline of the first action potential. On the other hand, repetitive stimulation had a clear effect on the steady levels of both the Ca and intrinsic signals: they summated.

Fig. 6 B shows optical traces similar to those in Fig. 6 A but taken with a reduced sampling rate and plotted on a slower time scale. The small fluctuations in the

$\Delta A(690)$ traces are due to changes in incident light intensity. These have been largely removed from the $\Delta A(570)$ traces [and from the $\Delta A(520)$ traces; not shown] by the intrinsic correction. The $\Delta A(690)$ signals, after either 1 or 10 action potentials, showed an early peak followed by a relatively constant plateau; the plateau level was maintained for the 8-s period of recording. The $\Delta A(570)$ signals were also maintained for 8 s, although the signal after 10 action potentials showed some recovery.
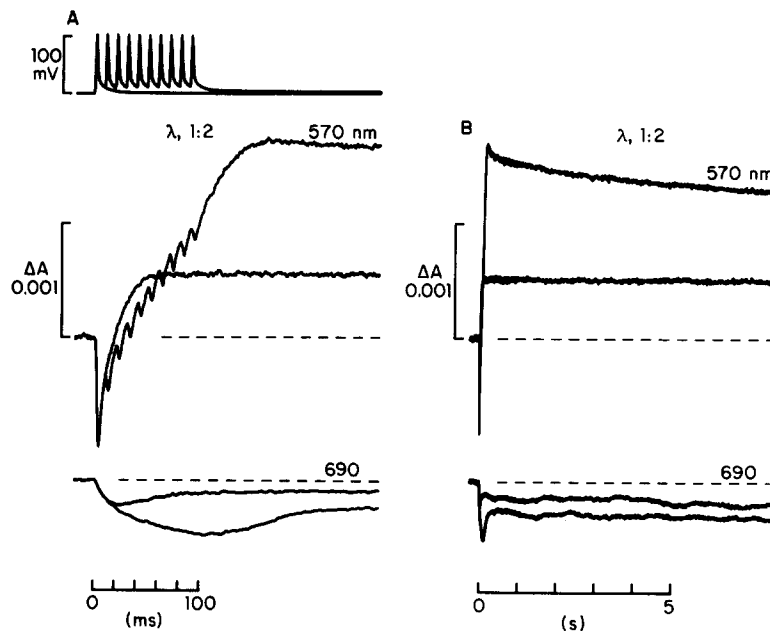

FIGURE 6.   Effect of repetitive stimulation on $\Delta A(570, 1:2)$ and $\Delta A(690, 1:2)$. (A) The top pair of traces shows the action potentials. The first action potential had an actual peak amplitude of 134 mV. The middle pair of traces shows $\Delta A(570, 1:2)$, corrected for the intrinsic contribution using $n = 1.0$. The bottom pair of traces shows $\Delta A(690, 1:2)$; the lower trace was obtained with a 10-action-potential stimulation. (B) Similar set of traces as in A but taken ~14 min later using a slower sampling rate. Fiber 101984.1; sarcomere spacing, 3.7 μm; indicator concentration, 3.86–3.87 mM in A and 3.97 mM in B. The traces in A were taken 87–89 min after saponin treatment; those in B were taken 101–103 min after saponin treatment.

Fig. 7 shows maintained levels of $\Delta A(570)$ (panel A) and $\Delta A(690)$ (panel B), plotted as a function of the number of action potentials during repetitive stimulation at 100 Hz. In both panels, the first action potential produced a much larger change than subsequent ones. The variability in the data in panel B arises from fluctuations in incident light intensity during the course of each measurement (see 690-nm traces in Fig. 6B).

The maintained components of the $\Delta A(570)$ tetramethylmurexide signal and the $\Delta A(690)$ intrinsic signal show two similarities: both signals summate during repetitive stimulation, with the largest change being produced by the first action

potential, and both signals are maintained for at least 8 s. These results raise the question of whether these two signals have a common origin, a possibility that deserves further consideration.

Relative Timing of the Action Potential, Ca, and Retardation Signals

Fig. 8 shows the action potential plus simultaneously recorded $\Delta A(570)$ and $\Delta R(690)$ signals obtained using mode 2 recording (Fig. 3 in Irving et al., 1987). The $\Delta A(570)$ trace, or Ca signal, reached half-way to peak 2.56 ms later than the action potential and had a half-width of 4.03 ms. The $\Delta R(690)$, or retardation, signal reached half-way to peak 1.45 ms later than $\Delta A(570)$ and had a considerably larger half-width, 26.3 ms.
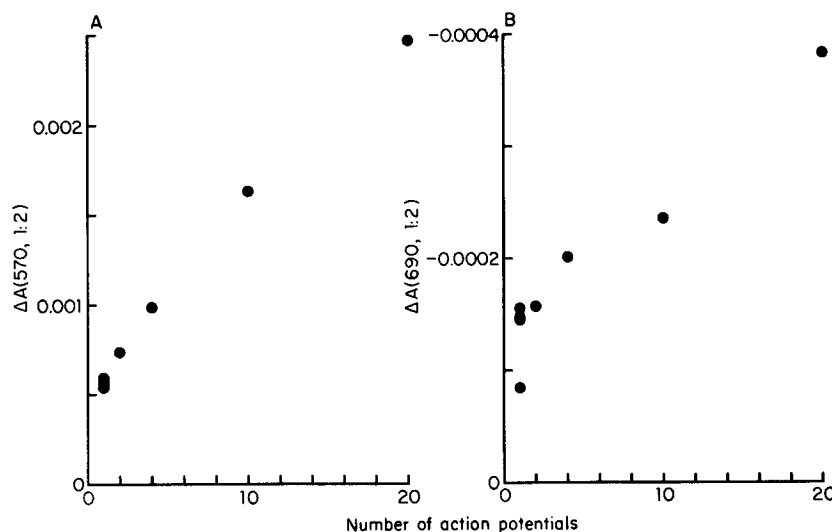

FIGURE 7. Maintained levels of $\Delta A(570, 1{:}2)$ (panel A) and $\Delta A(690, 1{:}2)$ (panel B) plotted against the number of action potentials after stimulation at 100 Hz. The values were measured ~0.4 s after the first action potential. Same experiment as in Fig. 6. Indicator concentration, 3.78–3.92 mM; time after saponin treatment, 82–93 min.

Columns 5–7 in Table III give information about $\Delta A(570)$ signals in four fibers in which $\Delta A(570)$ vs. $A(520)$ data were available (Fig. 5 and p. 157). One trace was selected from each fiber early in the experiment as soon as the indicator concentration reached 2–3 mM. The average time to half-peak was 2.73 ms and the average half-width was 4.48 ms. Since the early transient and maintained absorbance changes are of opposite sign and overlap, the half-width of $\Delta A(570)$ is not a reliable estimate of the half-width of myoplasmic free [Ca]; this half-width is probably greater than the values in column 6. The values of myoplasmic peak free [Ca] in column 7 were calculated on the assumption that only Ca-free tetramethylmurexide, given by 0.87 times the total amount, is available to react with myoplasmic Ca (see above). The average value was 17.0 μM. This is in good agreement with 16.7 μM (p. 157), obtained, using many experimental points, from the same fibers.

Separation of Transient and Maintained ΔA(570) Signals

This section explores the possibility that the tetramethylmurexide $\Delta A(570)$ signal consists of two components, a transient component that tracks myoplasmic free [Ca] and a maintained component. A method is described for estimating the waveform of the maintained component; this can then be subtracted from $\Delta A(570)$ to give the transient or myoplasmic component.

The maintained component represents a decrease in the amount of tetramethylmurexide that appears to be complexed with Ca at rest. It can be estimated,
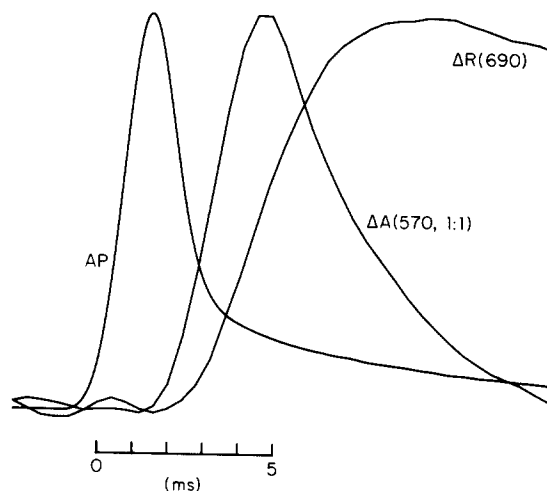

FIGURE 8. Relative time course of the action potential, $\Delta A(570, 1{:}1)$, and $\Delta R(690)$. A cubic spline function (Greville, 1968) was used to draw the curve that connects the digitized experimental points of the action potential. The points in the other traces were connected by straight line segments. The $\Delta A(570, 1{:}1)$ trace was corrected for the intrinsic contribution by using the $\Delta A(690, 1{:}1)$ trace with $n = 0$. The negative-going optical signals have been plotted with inverted polarity. All traces have been scaled to have the same peak. Same fiber as used in Fig. 6; resting retardation, 209 nm (average of three wavelengths); indicator concentration, 3.14 mM; time after saponin treatment, 65 min; temperature, 18.6°C. The waveform of the action potential was distorted by the 0.625-kHz eight-pole Bessel filter: the earliest electrical change precedes the $t = 0$ mark (determined by shifting the start of the stimulus 1.6 ms to the right to correct for the low-frequency delay of the Bessel filter) and the relative amplitude of the afterpotential is enhanced.

tentatively, on the assumption that these Ca-indicator complexes are inside the sarcoplasmic reticulum (see Discussion) and that some of them dissociate when Ca is released. Several changes in the sarcoplasmic reticulum may contribute to this dissociation: the volume may change, the internal free [Ca] may decrease, or indicator may move across the sarcoplasmic reticulum membrane. For present purposes, a detailed description of these events is not necessary; it suffices to know their effects. The effects are assumed to be that the maintained signal is due to Ca release from the sarcoplasmic reticulum, that it is directly proportional

to the cumulative amount of Ca released, and that it occurs without any added delay. Because the maintained signal lasts for at least 8 s, at least after a single action potential (Fig. 6B), it is also assumed that the signal is not reversed by Ca pump activity or other processes that occur within 1 s of stimulation.

The time course of sarcoplasmic reticulum Ca release was calculated using model 2 of Baylor et al. (1983); details are given in Maylie et al. (1987b) and in the legend to Fig. 11. Basically, the total concentration of myoplasmic Ca, $[Ca]_T$, is calculated as the sum of free Ca plus all the Ca that is complexed with indicator, parvalbumin, and the Ca-regulatory sites on troponin. After stimulation, $[Ca]_T$ rapidly increases as Ca is released from the sarcoplasmic reticulum. At later times, it slowly decreases as Ca is bound to the sarcoplasmic reticulum

TABLE III

Parameters Associated with Ca Signals Recorded with Tetramethylmurexide

(1)	(2)	(3)	(4)	(5)	(6) ΔA(570)	(7)	(8)	(9) Myoplasmic ΔA(570)	(10)
Fiber reference	Tetra-methyl-murexide	Time after saponin treatment	Time after indicator	Time to half-peak	Half-width	Peak free [Ca]	Time to half-peak	Half-width	Peak free [Ca]
	mM	min	min	ms	ms	μM	ms	ms	μM
061584.2	2.12	70	34	3.07	4.48	19.8	3.08	7.71	30.0
062784.1	1.96	61	30	2.86	4.45	17.5	2.93	8.65	27.1
071184.1	1.75	85	34	2.44	4.94	16.2	2.50	7.90	22.5
101984.1	3.14	65	32	2.56	4.03	14.3	2.64	7.50	23.0
Mean		70	33	2.73	4.48	17.0	2.79	7.94	25.7
SEM				0.14	0.19	1.2	0.13	0.25	1.8

Column 1 gives fiber reference. Column 2 gives the concentration of indicator at the site of optical recording. Column 3 gives the time that elapsed from saponin treatment to the measurement. Column 4 gives the time from application of indicator to the end pools to the measurement. Column 5 gives the interval between the time to half-peak of the action potential and the time to half-peak of indicator-related ΔA(570). Column 6 gives the half-width of the ΔA(570) signal. Column 7 gives the myoplasmic peak free [Ca] calculated from ΔA(570) using Eq. 1; in the calculation, $[TMX]_T$ was set equal to 87% of the total tetramethylmurexide, based on the assumption that all Ca-free tetramethylmurexide was able to react with myoplasmic Ca (see text). Columns 8–10 are similar to columns 5–7 except that they refer to the myoplasmic component of indicator-related ΔA(570); the procedure for estimating this component is described in the text and illustrated in Fig. 9. Average temperature, 18.1°C.

membranes and pumped back inside. The waveform of the maintained absorbance change is assumed to equal the waveform of cumulative Ca release, taken as the rising phase of $[Ca]_T$ up to time of peak and set constant to peak value at later times. Since a scaled release waveform is subtracted from ΔA(570) to give the myoplasmic component, and since the [Ca] transient determined by this myoplasmic ΔA(570) component usually produces a different release waveform, the procedure for estimating the maintained waveform requires iteration. Three to four times are usually sufficient to produce an invariant release waveform.

Fig. 9 shows the final result. In panel A, the top trace shows an action potential and the pair of traces below shows the original ΔA(570) signal and the estimated waveform of sarcoplasmic reticulum Ca release, scaled to match the final level

of the $\Delta A(570)$ signal. The action potential and $\Delta A(570)$ are the same as in Fig. 8. Panel B shows the myoplasmic component of the $\Delta A(570)$ signal, taken as the difference between the pair of waveforms in panel A. Myoplasmic components have also been estimated using models 1 and 3 of Baylor et al. (1983) to calculate Ca release (not shown); they are similar to that in Fig. $9B$. According to the above assumptions, the $\Delta A(570)$ trace in Fig. $9B$ represents the absorbance change associated with the myoplasmic free [Ca] transient.
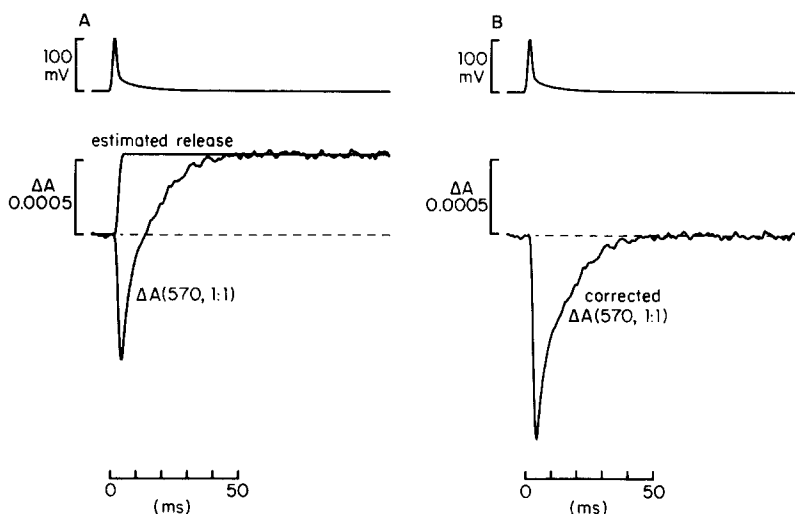

FIGURE 9.　Method for estimating the time course of the transient and maintained components of $\Delta A(570, 1:1)$. The essential assumptions are that the maintained component is directly proportional to the amount of Ca released from the sarcoplasmic reticulum and that this can be estimated, as described in the text, from calculations using model 2 of Baylor et al. (1983). (A) The top trace shows the action potential; actual amplitude, 134 mV. The next two traces show $\Delta A(570)$ and the estimated time course of sarcoplasmic reticulum Ca release, scaled to match the final level of the $\Delta A(570)$ signal. This scaled signal is the estimated maintained component of $\Delta A(570)$. (B) Action potential and the estimated transient component of $\Delta A(570)$, obtained by taking the difference between the two superimposed traces in panel A. Same fiber as used in Figs. 6–8; indicator concentration, 3.14 mM; time after saponin treatment, 65 min.

Columns 8 and 9 of Table III give timing information about myoplasmic $\Delta A(570)$ signals from four fibers. The values are similar to those obtained with antipyrylazo III (Table IV in Maylie et al., 1987b). The average time to half-peak was 2.79 ms for tetramethylmurexide and 3.07 ms for antipyrylazo III; the difference is not significant. The average half-width of the Ca signal was 7.94 ms with tetramethylmurexide and 10.10 ms with antipyrylazo III. These numbers are significantly different with $P < 0.025$ using the two-tailed t test. This difference is not due to differences in time after saponin treatment since these times were similar in the two sets of experiments; the average tetramethylmurex-

ide measurement was made 70 min after saponin treatment (Table III, column 3) and the average antipyrylazo III measurement was made 58 min after treatment (Table IV, column 2, in Maylie et al., 1987*b*).

Column 10 of Table III gives the myoplasmic peak free [Ca], calculated on the assumption that Ca-free indicator (fractional amount 0.87) is available to react with myoplasmic Ca (see p. 157). The average value was 25.7 μM. On the other hand, if only freely diffusible tetramethylmurexide (= $1/[R + 1]$ = 0.73) is available to react with Ca (see p. 157), the average value would be 30.6 μM.

Comparison of the Time Course of Myoplasmic Tetramethylmurexide and Antipyrylazo III Ca Signals

The preceding section showed that the myoplasmic Ca signal estimated with tetramethylmurexide is significantly briefer than that measured with antipyrylazo III. This raises the question of how rapidly antipyrylazo III tracks myoplasmic [Ca]. Baylor et al. (1985) provide information on this point. They injected an intact fiber with both antipyrylazo III and azo1, a new tetracarboxylate indicator (Tsien, 1983), and measured changes in absorbance after action potential stimulation. By choice of suitable wavelengths, they determined the Ca-related absorbance change of each indicator. The azo1 signal lagged the antipyrylazo III signal, which suggests that azo1 tracks free [Ca] with a greater delay than antipyrylazo III. To explain both indicator waveforms with a single free [Ca] waveform, it was necessary to assume that antipyrylazo III also tracks [Ca] with a delay. A good fit was obtained using a first-order filter with a time constant of 1/700 s.

Fig. 10 compares tetramethylmurexide and antipyrylazo III Ca signals on the assumption that tetramethylmurexide tracks free [Ca] instantaneously (see p. 148) and antipyrylazo III with a first-order delay of 1/700 s. The top pair of traces shows action potentials, scaled to the same peak amplitude, recorded from two different fibers, one that contained 0.306 mM antipyrylazo III and one that contained 3.14 mM tetramethylmurexide. The two waveforms are extremely similar. The next pair of traces shows the respective myoplasmic Ca signals. The trace labeled "ΔA(570, 1:1)," obtained with tetramethylmurexide, clearly leads the trace labeled "ΔA(720, 1:2)," obtained with antipyrylazo III. The bottom pair shows the tetramethylmurexide signal, filtered with a 1/700-s time constant, superimposed on the antipyrylazo III ΔA(720, 1:2) signal. The two traces are closely similar, particularly on the rising phase, which is consistent with tetramethylmurexide reacting almost instantaneously with Ca (Geier, 1968; Ogawa et al., 1980) and antipyrylazo III tracking myoplasmic Ca with a 1–2-ms first-order delay (Baylor et al., 1985).

A first-order filter of 1/700 s was applied to the transient component of ΔA(570) in the four experiments tabulated in Table III. The filter increased both the time to half-peak, on average from 2.79 to 3.61 ms (0.13 SEM), and the half-width, from 7.94 to 11.66 ms (0.39 SEM). Average values from fibers containing 0.3 mM antipyrylazo III lie within these ranges, with a time to half-peak of 3.07 ms and a half-width of 10.1 ms (Table IV in Maylie et al., 1987*b*).

The results in Figs. 9 and 10 show that the myoplasmic component of the tetramethylmurexide signal is similar to the antipyrylazo III Ca signal. This supports the idea that this component comes from tetramethylmurexide that is located in myoplasm and is able to react rapidly with myoplasmic Ca. A quanti-
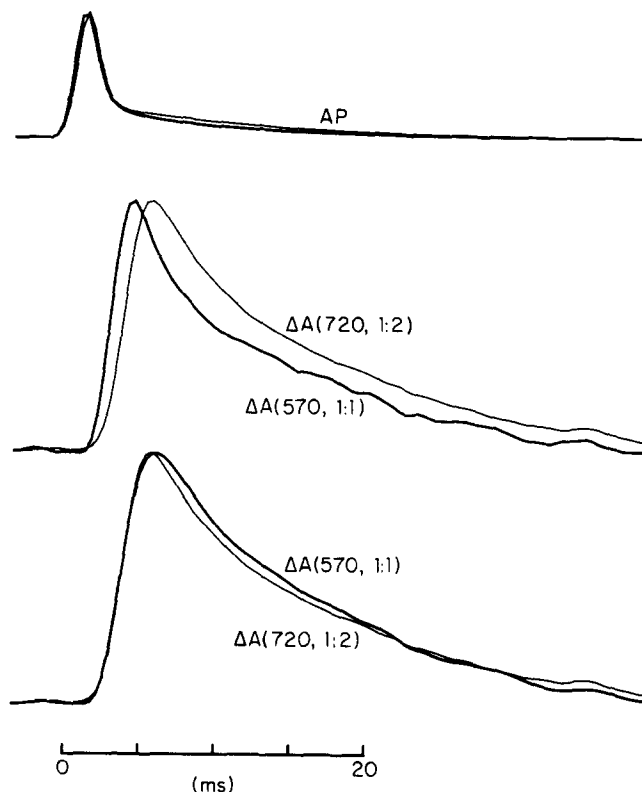

FIGURE 10. Comparison of the transient component of tetramethylmurexide absorbance and the antipyrylazo III Ca signal. Measurements were made on two different fibers, one containing 3.14 mM tetramethylmurexide (plotted with thicker line width) and another containing 0.306 mM antipyrylazo III. The top traces show the two action potentials scaled to peak; the actual amplitudes were 134 mV in the tetramethylmurexide-containing fiber and 130 mV in the antipyrylazo III fiber. The next pair of traces shows the transient component of $\Delta A(570, 1:1)$ from the tetramethylmurexide fiber and $\Delta A(720, 1:2)$ from the antipyrylazo III fiber, scaled to peak. The bottom traces show the $\Delta A(720)$ signal, repeated, and the $\Delta A(570)$ signal, delayed by a single time constant 1/700-s filter and rescaled; the filter reduced the actual peak amplitude by 20%. The $\Delta A(570)$ signal shown in the middle set of traces is the same one shown in Fig. 9B but plotted with reverse polarity; temperature, 18.6°C. The $\Delta A(720)$ signal is from a typical experiment with ~0.3 mM antipyrylazo III; same trace as shown in Figs. 5 and 21 of Maylie et al. (1987b); temperature, 19.2°C. The tetramethylmurexide and antipyrylazo III measurements were made 65 and 56 min, respectively, after saponin treatment.

tative comparison, illustrated in Fig. 10 and described in the preceding paragraph, further indicates that antipyrylazo III probably tracks myoplasmic Ca with a lag. Baylor et al. (1985) estimated a delay of 1/700 s. Our results are consistent with this or perhaps a somewhat smaller value.

DISCUSSION

To the best of our knowledge, the optical transients in this article are the only ones recorded from muscle fibers containing either murexide or tetramethylmurexide that have been published since Jobsis and O'Connor (1966). Our experiments were carried out on single frog fibers at ~18°C, whereas the Jobsis and O'Connor experiments used toad (*Bufo marinus*) sartorius muscles at 9–12°C. The two sets of results show important differences that seem surprising in view of the similarity of experimental material and conditions.

These differences concern the waveform of the Ca signal. The signals published by Jobsis and O'Connor were slow in onset and reached peak values 55–80 ms after stimulation; in contrast, the Ca signals in this article reached peak value ~5 ms after stimulation (Fig. 8). After the peak, the Jobsis and O'Connor Ca signals decayed slowly to baseline level, taking 110–150 ms after stimulation to decline to half-peak value. The Ca signals in this article decayed rapidly, reached baseline 10–20 ms after stimulation, and then reversed direction to a maintained level; this reversal was not observed by Jobsis and O'Connor. Another difference is that the peaks of the Jobsis and O'Connor Ca signals summated during tetanic stimulation, whereas the peaks in this article decreased (Fig. 6A). The most likely explanation for these differences is that toad and frog muscle have different properties or that the Jobsis and O'Connor Ca signals were contaminated by movement or some other artifact.

Binding and Sequestration of Tetramethylmurexide in Resting Fibers

Diffusion analysis (Fig. 1) indicates that, on average, 0.27 (given by $R/[R + 1]$, Table II, column 5) of the tetramethylmurexide inside muscle fibers is bound to or sequestered by myoplasmic constituents. Resting absorbance spectra (Fig. 2) show that, on average, 0.13 of the tetramethylmurexide inside resting fibers appears to be complexed with Ca. The aim of this section is to offer an explanation for these two findings.

Since the K_D of tetramethylmurexide for Ca is more than 10^4 times the resting free [Ca] in myoplasm, the most straightforward explanation of the resting spectrum is that at least 0.13 of the indicator is in a high-Ca environment. Although other possibilities should not be dismissed, the most likely such environment in muscle is the sarcoplasmic reticulum. This explanation is plausible since tetramethylmurexide can enter bullfrog fragmented sarcoplasmic reticulum vesicles in the presence of external ATP (Ogawa et al., 1980). Any indicator inside the sarcoplasmic reticulum, either Ca-free or Ca-complexed, would contribute to the amount of indicator that is estimated by diffusion analysis to be bound or sequestered.

For simplicity, one might assume that Ca-complexed indicator inside the sarcoplasmic reticulum is impermeant and that Ca-free indicator is in equilibrium

between myoplasm and the inside of the sarcoplasmic reticulum. If indicator inside the sarcoplasmic reticulum is the only source of binding or sequestration, 0.27 of the indicator inside muscle would be inside the sarcoplasmic reticulum and approximately half of this (given by 0.13/0.27) would be complexed with Ca. To account for this ratio, free [Ca] inside the sarcoplasmic reticulum would need to approximately equal the K_D of tetramethylmurexide, 2.6 mM in our calibration solutions at 15°C. This value is similar to estimates of sarcoplasmic reticulum free [Ca] made by Hasselbach and Oetliker (1983). The fractional volume of the sarcoplasmic reticulum, with respect to the combined volume of sarcoplasmic reticulum plus myoplasmic solution, should be given by the ratio (Ca-free indicator inside the sarcoplasmic reticulum)/(total Ca-free indicator) = (0.27 − 0.13)/(1 − 0.13) = 0.16. This number is similar to, although slightly larger than, morphological estimates obtained from electron-microscopic studies, 0.13 (Peachey, 1965) and 0.09 (Mobley and Eisenberg, 1975). This analysis assumes that the fraction of indicator that does not diffuse, 0.27, is inside the sarcoplasmic reticulum. It also assumes that the surface and transverse tubular membranes are impermeable to tetramethylmurexide, a point on which we have no direct information. If these assumptions are essentially correct, the sarcoplasmic reticulum explanation seems roughly consistent with both the observed diffusion of tetramethylmurexide and its resting absorbance inside muscle fibers.

Another explanation for the resting spectrum would be that some tetramethylmurexide molecules, not necessarily the fraction 0.13, are in a nonaqueous environment, an environment that changes either the absorbance of the indicator or its sensitivity to cations such as Ca. Ohnishi (1978) has shown that environment can affect the absorbance of tetramethylmurexide; the absorbance spectrum of tetramethylmurexide at pH 7 in a solution containing 95% methanol, 5% water, and no Ca is similar to that in 100% water and 1 mM Ca. If this kind of effect underlies the shape of the resting spectrum, it might also account for some or all of the indicator that appears to be bound or sequestered.

Other explanations should also be considered as well as the possibility that more than one process is involved. For example, some tetramethylmurexide molecules may be inside the sarcoplasmic reticulum, others may be sequestered elsewhere such as in mitochondria, and still others may be in a nonaqueous environment of the type described in the preceding paragraph.

Binding of Ca to Myoplasmic Sites after Action Potential Stimulation

After action potential stimulation, the change in absorbance of intracellular tetramethylmurexide can be separated into two components, an early transient or myoplasmic component and a maintained component. In this section, the myoplasmic component of the tetramethylmurexide $\Delta A(570)$ signal (Fig. 9B) is used to estimate the waveform of myoplasmic free [Ca]. The time course of Ca binding to troponin and parvalbumin can then be calculated (Fig. 11) according to the method used in Figs. 21–24 in the preceding article (Maylie et al., 1987b); details are given in the legend to Fig. 11 and in model 2 of Baylor et al. (1983).

The bottom trace in Fig. 11 shows the time course of myoplasmic Ca:tetramethylmurexide concentration estimated from the $\Delta A(570)$ signal in Fig. 9B.

The next trace up shows free [Ca] obtained from Eq. 1. The next four traces show [CaTrop], the concentration of Ca-regulatory sites on troponin that are complexed with Ca; [MgParv], the concentration of Ca,Mg sites on parvalbumin complexed with Mg; [CaParv], the concentration of these parvalbumin sites complexed with Ca; and total myoplasmic Ca, $[Ca]_T = [Ca] + [CaTMX] + [CaTrop] + [CaParv]$. As described above, the first part of the $[Ca]_T$ trace was used in Fig. 9A for the time course of Ca release from the sarcoplasmic reticulum. The trace second from the top shows the net rate of movement of Ca from
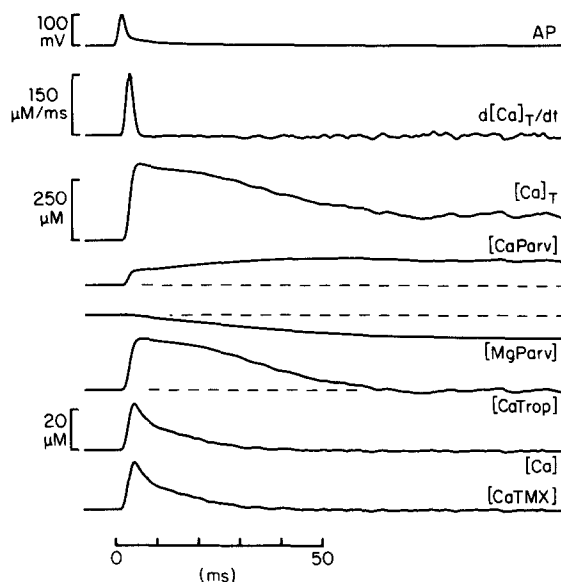

FIGURE 11. Modeling of Ca movements after a single action potential, using the transient component of the tetramethylmurexide signal. The bottom trace shows the time course of myoplasmic [CaTMX] determined from the transient component of $\Delta A(570, 1:1)$ in Fig. 9B. For the determination, the prestimulus baseline was set constant to its average value; this level corresponds to the assumed value of 0.02 μM for resting free [Ca]. The next trace up shows free [Ca] calculated from Eq. 1, using the parameters given in the Methods and assuming that 0.87 of the tetramethylmurexide was available to react with Ca. The 20-μM calibration mark applies to the lower two traces. The next four traces (250 μM calibration mark) show Ca complexation with troponin ([CaTrop]); Mg and Ca complexation with parvalbumin ([MgParv] and [CaParv]); and total Ca ($[Ca]_T = [Ca] + [CaTMX] + [CaTrop] + [CaParv]$). The next trace up shows the time derivative of $[Ca]_T$ and the top trace shows the action potential. The calculations were carried out using model 2 of Baylor et al. (1983), except that free [Mg] was set to 1 instead of 2 mM. The myoplasmic concentration of Ca-regulatory sites on troponin was taken as 240 μM, the K_D as 2 μM, the forward rate constant as 0.575×10^8 M^{-1} s^{-1}, and the backward rate constant as 115 s^{-1}. The concentration of Ca,Mg sites on parvalbumin was taken as 1 mM, the K_D for Ca as 4 nM (with the forward rate constant equal to 1.25 $\times 10^8$ M^{-1} s^{-1} and the backward rate constant equal to 0.5 s^{-1}), and the K_D for Mg as 91 μM (with the forward rate constant equal to 3.3×10^4 M^{-1} s^{-1} and the backward rate constant equal to 3.0 s^{-1}).

sarcoplasmic reticulum to myoplasm, $d[Ca]_T/dt$, and the top trace shows the action potential.

Calculations were carried out on the four experiments tabulated in Table III. They gave the following average values (± SEM):

Free [Ca]: time to half-peak = 2.80 ± 0.13 ms; half-width = 7.88 ± 0.25 ms; peak value = 25.7 ± 1.8 μM.

[CaTrop]: time to half-peak = 3.07 ± 0.09 ms; peak value = 216.0 ± 1.8 μM. $[Ca]_T$: peak value = 317.2 ± 5.0 μM.

$d[Ca]_T/dt$: time to half-peak = 1.99 ± 0.12 ms; half-width = 1.86 ± 0.07 ms; peak value = 163.4 ± 6.4 μM/ms; minimum value = −4.32 ± 0.54 μM/ms.

The times to half-peak are referenced to the time to half-peak of the action potential. Since peak free [Ca] is two orders of magnitude smaller than the K_D of tetramethylmurexide for Ca, free [Ca] is linearly related to $\Delta A(570)$, and the values of time to half-peak and half-width of free [Ca] are similar to the corresponding values for myoplasmic $\Delta A(570)$ (Table III, columns 8 and 9). Since the properties of the free [Ca] waveform are similar to those obtained with antipyrylazo III using the high Ca calibration, the values of parameters associated with [CaTrop], $[Ca]_T$, and $d[Ca]_T/dt$ are almost equal to the corresponding values given in Table VII in Maylie et al. (1987b). Hence, much of the discussion about antipyrylazo III Ca transients, using the high Ca calibration, in the preceding article applies equally well here.

Relative Timing of the Action Potential and Three Ca-related Signals

Fig. 12 shows the action potential, $d[Ca]_T/dt$, free [Ca], and [CaTrop] from Fig. 11. The traces have been scaled to the same peak and plotted on an expanded time base. Since the waveform of myoplasmic free [Ca] is closely similar to that estimated with antipyrylazo III using the high Ca calibration (Maylie et al., 1987b), apart from the 1–2-ms delay introduced by antipyrylazo III (Fig. 10 and Baylor et al., 1985), the relative comparison of waveforms in Fig. 12 is similar to that in Fig. 24B of Maylie et al. (1987b). Average values for various timing parameters are given in the preceding section.

After the action potential (Fig. 12), the first waveform is that of $d[Ca]_T/dt$, the calculated net Ca flux from the sarcoplasmic reticulum into myoplasm. Its average time to half-peak, after that of the action potential, was 2 ms. This includes the time required for the action potential to spread radially into the transverse tubular system and for the Ca release mechanism to be activated. The half-width of the Ca flux waveform was slightly less than 2 ms, approximately the same as that of the action potential. Thus, the regulation of sarcoplasmic reticulum Ca release by the potential across the transverse tubular membranes must be extremely rapid for both activation and deactivation.

The waveforms of free [Ca] and [CaTrop] begin slightly after $d[Ca]_T/dt$ and reach half-peak 0.8 and 1.1 ms later, respectively. After [CaTrop] reaches a peak, it remains elevated until [Ca] approaches the K_D of the Ca-regulatory sites on troponin, which in these calculations was 2 μM. Ca binding to the Ca-regulatory sites on troponin precedes the change in intensity of the second actin layer line, an early X-ray signal that has been associated with movement of tropomyosin on the surface of the thin filaments. At 18°C, the time to half-peak

of the X-ray signal occurs ~6 ms after that of the action potential (Kress et al., 1986) or ~3 ms after that of [CaTrop]. Thus, the rate constants used to calculate the binding of Ca to Ca-regulatory sites on troponin are sufficiently fast that the predicted binding of Ca to troponin can account for the earliest change in the X-ray diffraction pattern.
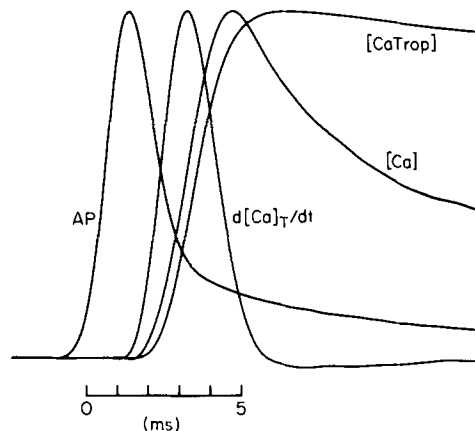

FIGURE 12. Relative timing of the action potential and three Ca-related signals. The traces of action potential, $d[Ca]_T/dt$, [Ca], and [CaTrop] from Fig. 11 have been plotted using a cubic spline function (Greville, 1968) to connect the points. The traces were scaled to have the same peak. Temperature, 18.6°C.

Amplitude of the Myoplasmic Free [Ca] Transient Estimated with Tetramethylmurexide

The results in this article provide the first direct evidence that free [Ca] inside skeletal muscle fibers increases to a level as high as 17 μM, on average, after a single action potential (Table III, column 7). This estimate, however, is probably too low for two reasons: it is calculated from the peak value of the total $\Delta A(570)$ signal, rather than from the transient component, and it assumes that all the Ca-free indicator is able to react with myoplasmic Ca. If the transient component is separated from the total signal, using the procedure described above (Fig. 9), the estimated peak [Ca] is increased to 26 μM (Table III, column 10). If some of the Ca-free indicator is not in the myoplasmic solution, for example, if it is inside the sarcoplasmic reticulum, it would be further increased. If only freely diffusible indicator can react with Ca, the estimate would be 31 μM. Hence, it seems likely that peak free [Ca] after a single action potential is 20–30 μM.

An assumption underlying all these estimates is that tetramethylmurexide is well behaved inside a fiber and can complex Ca with the same affinity and same change in absorbance that occur in a calibration solution. Although there is no direct evidence on this point, it seems reasonable since a large fraction of indicator is freely diffusible.

Several physiologically interesting consequences follow if the amplitude of the free [Ca] transient is indeed 20–30 μM. The first is that, during a brief tetanus,

the plateau level of [Ca] should equal 10–20 μM (see below) and a fraction 0.8–0.9 of the Ca-regulatory sites on troponin should be complexed with Ca (using K_D = 2 μM as given by Potter and Zot, 1982, and Rosenfeld and Taylor, 1985). This almost maximum fractional occupancy is consistent with observations on intact frog muscle fibers that indicate that nearly maximum activation is achieved during a brief tetanus at the temperatures used in our experiments (Blinks et al., 1978; Lopez et al., 1981) and with observations on isolated rabbit myofibrils that indicate that maximum activation of tension or Mg-ATPase activity requires that Ca be bound to all the Ca-regulatory sites on troponin (Fuchs and Fox, 1982; Blanchard et al., 1984).

A second consequence of a 20–30-μM [Ca] transient is that, in spite of its brief duration, with a half-width of 8 ms after a single action potential at 18°C, the transient is able to complex a fraction 0.9 of the Ca-regulatory sites on troponin (using the rate constants in the legend to Fig. 11). This is consistent with the peak intensity change of the second actin layer line being almost as large after a single action potential as during a brief tetanus (Kress et al., 1986).

A third consequence of a large [Ca] transient is that a high level of free [Ca] would be maintained during a tetanus. According to the results with antipyrylazo III (Table V, column 7, in Maylie et al., 1987b), the average level during a 0.1-s tetanus, with 100 Hz stimulation, is 0.8 times the peak produced by the first action potential. But since antipyrylazo III may track free [Ca] with a first-order delay of 1/700 s (Baylor et al., 1985), the estimate of peak [Ca] produced by the first action potential may be too low. Such a delay reduces the peak amplitude of a tetramethylmurexide Ca signal after a single action potential by 20% (see legend to Fig. 10). On this basis, any estimate of peak free [Ca], after a single action potential, obtained with antipyrylazo III would be only 0.8 times the actual value. With tetramethylmurexide, then, the level of [Ca] during a brief tetanus should be ~0.8 × 0.8 times the 20–30-μM peak value, or ~10–20 μM.

Somlyo et al. (1981) showed that a 1.2-s tetanus at 23–25°C does not produce statistically significant changes in the Ca content of frog muscle mitochondria. Since mitochondria accumulate Ca if cytoplasmic free [Ca] is sufficiently elevated, the negative results should in principle set an upper limit to the level of [Ca] that could be maintained during a 1.2-s tetanus. The rate of Ca uptake by isolated mitochondria depends on the source. As far as we know, measurements have not been made on mitochondria from frog muscle, but rates from mitochondria isolated from other tissues usually lie within the range 0.5–2 mmol/s·kg dry wt for free [Ca] = 10–20 μM in the presence of millimolar amounts of [Mg], 23–25°C (Scarpa and Granziotti, 1973; Scarpa, 1979). The standard error of the mean of the measurements of Somlyo et al. (1981) was 0.5–0.6 mmol/kg dry wt, so that a 1.2-s period of free [Ca] = 10–20 μM, coupled with the above rates of 0.5–2 mmol/s·kg dry wt for Ca uptake, would produce an increase in mitochondrial Ca just at or slightly above the level of detectability. Thus, the absence of detectable mitochondrial Ca uptake is not clearly inconsistent with a 10–20-μM level of [Ca] during a brief tetanus. Before this can be definitely settled, it will be necessary to know (a) whether the 10–20-μM plateau level of [Ca] observed

after 0.1 s of repetitive stimulation is maintained for a 1.2-s period, and (*b*) the rate of Ca uptake by mitochondria inside frog muscle with myoplasmic free [Ca] equal to the maintained tetanic level.

Origin of the Maintained Tetramethylmurexide Absorbance Change

The transient change in tetramethylmurexide absorbance, which probably tracks the increase in myoplasmic free [Ca], is quickly followed by a maintained change of opposite sign (Figs. 3 and 9). This is due to a change in absorbance of the indicator molecules that appear to be complexed with Ca at rest. Consequently, the origin of the maintained signal depends on the explanation of the resting spectrum.

If the Ca-like shape of the resting spectrum comes from Ca-bound indicator inside the sarcoplasmic reticulum, the maintained signal has a natural explanation in terms of Ca dissociating from these indicator molecules. Such dissociation might occur when Ca is released from the sarcoplasmic reticulum. Since the first action potential releases substantially more Ca than subsequent ones (Fig. 15 in Baylor et al., 1983; Fig. 23 in Maylie et al., 1987*b*), the first action potential should also produce a larger maintained signal. The results in Fig. 7 show that this is the case. Thus, the sarcoplasmic reticulum explanation is qualitatively consistent with the experimental results on repetitive stimulation.

An estimate of the change in free [Ca] inside the sarcoplasmic reticulum can be obtained in the following way. Suppose that neither the volume of the sarcoplasmic reticulum nor its total content of indicator changes during the first 0.1–0.2 s after stimulation. As mentioned on p. 157, the maintained absorbance change after one action potential corresponds to a decrease of 0.0030 in the fractional amount of tetramethylmurexide complexed with Ca. If this occurs inside the sarcoplasmic reticulum, and if this space accounts for the nondiffusing fraction of indicator, then the fractional change of the Ca:tetramethylmurexide complex inside the sarcoplasmic reticulum is given by $-0.0030/0.27 = -0.011$. The corresponding change in sarcoplasmic reticulum free [Ca] is given by the differential form of Eq. 1:

$$\Delta[Ca]/K_D = (1 + [Ca]/K_D)^2 \cdot \Delta[CaTMX]/[TMX]_T. \tag{2}$$

Using resting sarcoplasmic reticulum $[Ca] = K_D$, $\Delta[CaTMX]/[TMX]_T = -0.011$, and $K_D = 2.6$ mM gives $\Delta[Ca] = -114$ μM.

If the sarcoplasmic reticulum Ca concentration decreases by this amount because of release into myoplasm, the increase in the myoplasmic Ca concentration can be predicted from the ratio of sarcoplasmic reticulum volume to myoplasmic volume. The volume of the sarcoplasmic reticulum was estimated above, using simplifying assumptions, to equal 0.16 times the sum of the two volumes, giving a ratio of $0.16/0.84 = 0.19$. Thus, myoplasmic Ca should increase by $0.19 \times 114 = 22$ μM. This calculation uses the change in free sarcoplasmic reticulum [Ca] only. The decrease in total sarcoplasmic reticulum Ca, and consequently the estimated increase in total myoplasmic Ca, should be larger owing to the presence of sarcoplasmic reticulum Ca buffers such as calsequestrin. But whether these buffers are present in sufficient quantity to increase the above

estimate to equal the calculated increase in total myoplasmic Ca, 0.3 mM (Fig. 11 and p. 168), is not clear.

Alternatively, some of the simplifying assumptions, or perhaps even the sarcoplasmic reticulum hypothesis itself, may be incorrect. If the Ca-like shape of the resting spectrum is due not to Ca complexation but to tetramethylmurexide being in a nonaqueous environment (see above), one would need to assume that after stimulation there is a change in either the amount of indicator inside the environment or the properties of the environment.

Whatever the explanation for the maintained absorbance change, the experimental finding that is perhaps most puzzling is that the change, like the slow components of the change in intrinsic absorbance (Fig. 6B) or retardation (unpublished observations), is maintained for several seconds. By this time, according to the published rate constants used in our calculations, Ca should have dissociated from the Ca-regulatory sites on troponin (Fig. 11) and the Ca, Mg sites on parvalbumin (not shown). A considerable fraction of the released Ca should have returned to the terminal cisternae of the sarcoplasmic reticulum (since almost half returns by 0.5 s after a 1.2-s tetanus at room temperature; Somlyo et al., 1982), and the liberation of activation heat should be completed (Rall, 1979). Thus, within several seconds after an action potential, myoplasmic free [Ca] and Ca complexation by buffers should have returned to resting values. Yet changes in optical properties, and possibly the state of internal Ca, are well maintained for more than 8 s at 18°C.

The Need for a Better Ca Indicator for Skeletal Muscle

Several characteristics are required of an indicator if it is to measure myoplasmic [Ca] transients well (see Rakowski et al., 1985, for a discussion of how to measure Ca entry into myoplasm, rather than free [Ca], with indicators). Besides being easy to use and inexpensive to obtain, the indicator should show little response to changes in pH or [Mg] and should react rapidly with Ca. Once inside the fiber, the indicator should be confined to myoplasm and behave chemically and optically in the same way that it does in a cuvette calibration. If the optical signal is to be proportional to the spatial average of myoplasmic [Ca], the indicator should not be localized within the myoplasm and the stoichiometry of the Ca:indicator complex should be 1 Ca:n indicator molecules, 1:1 stoichiometry being the simplest example. To ensure that the optical response is everywhere approximately proportional to free [Ca], most of the indicator at any location should remain Ca-free. This requires that the apparent dissociation constant of indicator for Ca should be an order of magnitude greater than the largest value of free [Ca] to be encountered anywhere in the myoplasm. Since free [Ca] near release sites may reach a level several times as high as the average concentration (Cannell and Allen, 1984), a good 1:1 indicator for Ca in muscle, if it is to measure a 20–30-μM [Ca] transient, should have a K_D of at least 1 mM. These, then, are the main characteristics to look for in an indicator to be used to measure [Ca] transients in skeletal muscle.

Unfortunately, problems exist with all Ca indicators tried thus far in frog muscle, and one can only wonder whether similar problems arise when these indicators are used in other types of cells. Some of the difficulties encountered

with arsenazo III, antipyrylazo III, and tetramethylmurexide have been documented in this and the preceding two articles. Preliminary experiments with murexide indicate problems similar to those of tetramethylmurexide. Optical signals from dichlorophosphonazo III are highly complex and difficult to interpret (Baylor et al., 1982a, b). Aequorin has several advantages but is also not ideal. Its luminescence signals show significant delays (Hastings et al., 1969; Moore, 1984) and depend on [Ca] raised to the 2.5 power (Allen and Blinks, 1979). In addition, it has been shown to bind to skinned myocardial fibers (Fabiato, 1985).

Much interest has focused recently on the new class of tetracarboxylate indicators introduced by Dr. R. Y. Tsien. The two best-known examples are quin2 (Tsien, 1980) and fura-2 (Grynkiewicz et al., 1985). These compounds have apparent K_D's below 1 μM and would therefore not be expected to be useful in measuring the large Ca transients that occur in skeletal muscle. They may successfully measure resting levels, however.

To our knowledge, azo1 (Tsien, 1983) is the only tetracarboxylate indicator that has been used in skeletal muscle. Although its dissociation constant for Ca, 3.7 μM at 20°C (Hollingworth and Baylor, 1986), is an order of magnitude larger than that of quin2 or fura-2, it is still small compared with our estimate of the amplitude of the myoplasmic free [Ca] transient. As mentioned on p. 163, Baylor et al. (1985) simultaneously injected both azo1 and antipyrylazo III into a single muscle fiber and measured the two Ca signals by wavelength separation. The azo1 Ca signal lagged the antipyrylazo III signal, probably because azo1 reacts with myoplasmic free Ca more slowly than antipyrylazo III. After allowing for this reaction delay, the peak amplitude of the free [Ca] transient, assuming that all the azo1 could react normally with Ca, was estimated to be only 1–2 μM (Baylor et al., 1985; Hollingworth and Baylor, 1986). In intact fibers at 16°C, the apparent myoplasmic diffusion constant of azo1 is small, 0.10×10^{-6} cm^2/s, and slightly smaller than values obtained for arsenazo III, 0.21×10^{-6} cm^2/s, and antipyrylazo III, 0.12×10^{-6} cm^2/s (Baylor et al., 1986). Since the molecular weight of azo1 is smaller than that of the other two indicators, it seems likely that azo1 binds to or is sequestered by intracellular constituents at least as well as arsenazo III or antipyrylazo III. Such binding introduces uncertainty into the calibration of the Ca signal.

Thus, at least seven different Ca indicators have been tried in frog twitch fibers and a completely satisfactory one has yet to be found. Estimates of peak myoplasmic free [Ca] after an action potential range from 1 to 40 μM. In our opinion, the best estimate, 20–30 μM, is obtained with tetramethylmurexide. Unfortunately, with this indicator, the absorbance change produced by the myoplasmic Ca transient is contaminated by the maintained component, so that the time course of myoplasmic [Ca] cannot be determined with certainty. Since a quantitative understanding of how Ca participates in muscle activation requires a reliable estimate of myoplasmic free [Ca], it is important that new indicators become available. Good candidates might be found in low-affinity tetracarboxylate indicators, membrane-impermeant analogues of murexide (if its main problem is associated with entry into the sarcoplasmic reticulum), or other indicators having a K_D in the millimolar range.

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