COMPARISON OF ISOTROPIC CALCIUM SIGNALS FROM INTACT FROG MUSCLE FIBERS INJECTED WITH ARSENAZO III OR ANTIPYRYLAZO III

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ABSTRACT Intact single skeletal muscle fibers were micro-injected with either of the metallochromic indicator dyes Arsenazo III or Antipyrylazo III, and dye-related Ca^{2+} signals from each were measured during a twitch. In comparison with the Arsenazo III Ca^{2+} signal, the signal from Antipyrylazo III had three favorable features: (*a*) it was temporally faster, (*b*) its spectral dependence agreed with a cuvette calibration, and (*c*) its kinetic behavior was consistent with a single Ca^{2+}-dye stoichiometry. It is therefore suggested that the Antipyrylazo III Ca^{2+} signal is a more accurate monitor of the time course of the underlying myoplasmic free Ca^{2+} transient and one that may be more reliably calibrated.

INTRODUCTION

The experiments described in this paper were carried out for the purpose of evaluating the relative merits of the metallochromic dyes Arsenazo III and Antipyrylazo III as intracellular Ca^{2+} indicators in intact frog skeletal muscle fibers. In previous work on both intact and cut fibers, it was shown that, following introduction of either dye into myoplasm, a large dye-related absorbance change can be measured associated with muscle activation (Miledi et al., 1977, 1981, 1982; Baylor et al., 1979, 1982*b*,*c*; Kovács et al., 1979, 1983; Palade and Vergara, 1981, 1982*b*; Schneider et al., 1981). However, a detailed analysis of the spectral and polarization properties of the optical signals from intact fibers revealed some unanticipated features. For example, the absorbance signal from both dyes was shown to consist of a calcium-related component plus a component not directly due to the formation of calcium-dye product(s) (Baylor et al., 1982*b*,*c*). In particular, the Antipyrylazo III signal, examined at a series of wavelengths, was shown to have a component with the spectral dependence characteristic of a change in H^+ and/or Mg^{2+}; whereas the Arsenazo III signal, examined using two forms of linearly polarized light, was shown to have an anisotropic (or dichroic) component, which was particularly prominent when dye concentrations in myoplasm were less than ~0.5 mM.

Because of these and other complexities, it was of interest to carry out detailed measurements in intact fibers comparing the signals from each of the two dyes under identical experimental conditions. (Previous dye comparisons had been made at different temperatures or using different methods of dye exposure, see Baylor et al., 1982*b*; Palade and Vergara, 1982*b*.) If the calcium-related components measured under identical conditions were found to have similar properties, the degree of confidence in the information obtained about calcium would be strengthened. On the other hand, if significant differences were found, caution in the interpretation of the results would be suggested until the source of the differences could be explained.

METHODS

The methods employed were similar to those described in Baylor and Oetliker (1977) and Baylor et al. (1982a). Briefly, an intact, single, twitch fiber of the frog (*Rana temporaria*) was dissected from either the semitendinosus or iliofibularis muscle and mounted horizontally on an optical bench. The bathing solution was normal Ringer's (120 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 5 mM PIPES, pH = 7.10) at a temperature of 15–17°C. To minimize movement artifacts in the optical records, the fiber was stretched to a long sarcomere spacing (3.4 to 4.5 μm) and lowered onto a three-pedestal support. Purified dye was pressure injected into the fiber after impalement by a micro-electrode filled with either 15 mM Arsenazo III (Sigma Chemical Co., St. Louis, MO) or Antipyrylazo III (ICN K & K Laboratories Inc., Plainview, NY), titrated to pH 7.0. To measure fiber absorbance, a small region in the middle of the cell near the site of injection was illuminated with a circle of light smaller than the fiber diameter. Information by wavelength was obtained by inserting one of a series of narrow-band interference filters between the tungsten-halogen light source and the fiber. Information by polarization was obtained by splitting the quasi-monochromatic transmitted light beam into its 0° (electric vector parallel to the fiber axis) and 90° (electric vector perpendicular to the fiber axis) components, the intensities of which were

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simultaneously measured by two separate photodiodes. A single propagated action potential, and thereby fiber activity, was initiated by means of a brief (<1 ms) shock from a pair of extracellular electrodes positioned close to the site of optical recording. Any remaining twitch response was recorded by a sensitive tension transducer (model AE802, Aksjeselskapet Micro-Electronikk, Horten, Norway) attached to one tendon end of the fiber. Records were obtained from only those fibers showing stable, all-or-nothing optical signals. All data were sampled, stored and analyzed on a PDP-11 laboratory computer (Digital Equipment Corp., Marlboro, MA). To aid interpretation of the dye-related absorbance measurements from a fiber, their spectral and polarization properties were compared with in vitro cuvette calibration curves obtained using appropriate dye concentrations (e.g., 0.03 to 1.0 mM) and ionic conditions (e.g., 150 mM KCl, 1 to 2 mM Mg^{2+}, 5 to 10 mM PIPES at pH=6.90, with or without added Ca^{2+}).

RESULTS AND DISCUSSION

In Fig. 1, absorbance changes measured during activity from a fiber injected with Arsenazo III (*A*) are compared, as a function of wavelength and polarization, with those from a second fiber injected with Antipyrylazo III (*B*). For each dye the particular wavelengths shown were selected because of the spectral properties of the Ca^{2+}-dye difference spectrum measured in cuvette (see continuous curves, Fig. 2). In particular, at the longest wavelengths in Fig. 1 (750 nm for Arsenazo III; 810 nm for Antipyrylazo III), no dye-related signal is expected. Therefore, measurements at these wavelengths assess the magnitude of any residual movement artifacts or intrinsic absorbance changes in the optical records. For the two highly immobilized fibers in Fig. 1, it is apparent that these sources of error are, to a first approximation, negligible. If necessary, however, the intrinsic effects, which are also present in the records taken at a shorter wavelength λ_1, can be corrected, for example, by subtracting the signal measured at the long wavelength λ_0 scaled up by the ratio $(\lambda_0/\lambda_1)^2$.

The other wavelengths used in Fig. 1 correspond to maxima, minima, or null points (also called isosbestic points) in the Ca^{2+}-dye difference spectra. At early times after stimulation these signals vary in amplitude approximately as expected for an absorbance change due to the formation of Ca^{2+}-dye complex(es). In particular, large changes with similar time course are observed at 530, 600, and 660 nm (for Arsenazo III) and at 550, 650, and 720 nm (for Antipyrylazo III), whereas small to negligible changes are seen near the isosbestic wavelengths (~570 nm for Arsenazo III and ~590 nm for Antipyrylazo III). Furthermore, the superimposed 0°- and 90°-absorbance records taken at each wavelength are closely similar. This similarity is to be expected if the signals arise from activity in molecules that are primarily in the myoplasmic solution and not bound to oriented intracellular structures (for example, myofilaments or the sarcoplasmic reticulum). The small differences detectable by polarization in Fig. 1 (for example, at 570 nm for Arsenazo III and at 550 nm for Antipyrylazo III) are reproducible dye-related effects, which in the case of Arsenazo III have been analyzed in considerable detail (Baylor et al., 1982*c*). The remainder of

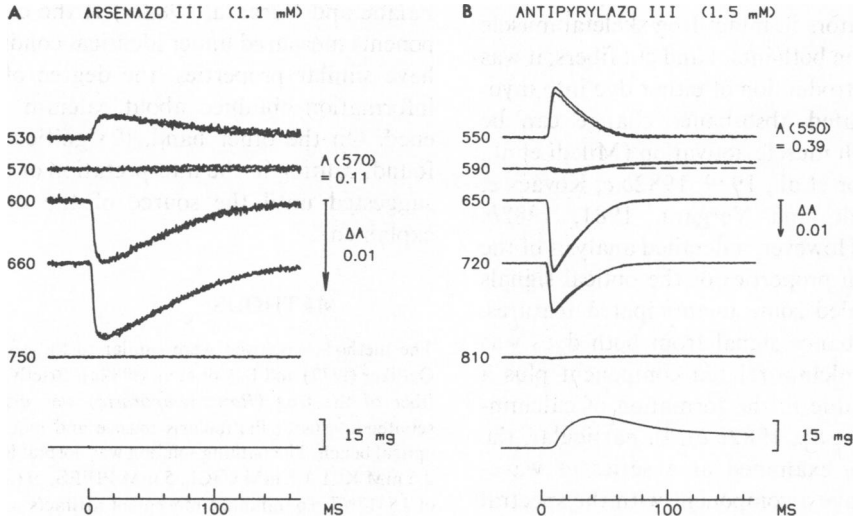

FIGURE 1 Original records of polarized absorbance changes (*upper* traces) and tension (*lowest* traces) in response to a single action potential initiated by an external shock at zero time. At each wavelength (indicated in nanometers to the left of the optical records), the 0°- and 90°-absorbance records taken simultaneously are superimposed. The records at different wavelengths were taken sequentially, in response to single shocks separated by 0.2 to 1.0 min. Dye concentrations were estimated using Beer's law: $A = \epsilon cl$, where $\epsilon(570) = 3 \times 10^4\ M^{-1}cm^{-1}$ was used for Arsenazo III and $\epsilon(550) = 2.55 \times 10^4\ M^{-1}cm^{-1}$ was used for Antipyrylazo III, and l is the pathlength through the entire fiber. Resting dye absorbance A was calculated from $A = \log_{10}(J/I)$, where J is the (estimated) light intensity transmitted by the fiber in the absence of dye and I in the presence of dye, whereas $\Delta A = -(\Delta I/I)/\log_e 10$. An upward deflection in the optical traces corresponds to an increase in transmitted light or a decrease in fiber absorbance. (*A*) Arsenazo III-injected fiber. Each trace shown is a single sweep. At later times the 90° record is detectably above the 0° record in the 530- and 570-nm traces. Fiber 061282.2; diameter, 34 μm; 16.3°C; sarcomere spacing, 4.3 μm. (*B*) Antipyrylazo III-injected fiber. Each trace shown is a single sweep, except for the 550- and 810-nm traces, which were signal-averaged twice. At early times the 90° record is detectably below the 0° record in the 550 and 590-nm traces, whereas it is very slightly above the 0° record in the 650- and 720-nm traces. Fiber 061082.2; diameter, 104 μm; 17.1°C; sarcomere spacing, 3.4 μm.

this paper is concerned with the isotropic components of the dye signals, defined as the weighted average, $(\Delta A_0 + 2\Delta A_{90})/3$, of the individual 0°- and 90°-absorbance changes. (Note, as shown in Baylor et al. [1982c], this averaging procedure provides a general way to eliminate absorbance changes due simply to a change in orientation of anisotropic dye molecules in the radially symmetric muscle fiber. However, for the experiments shown in Fig. 1 this correction makes an insignificant difference in the analysis that follows.)

Fig. 2 examines in detail the wavelength dependence of the early isotropic dye signals (open squares) from the fibers shown in Fig. 1. The continuous curve in *A* is a scaled Ca^{2+}-Arsenazo III difference spectrum obtained in cuvette, showing the wavelength dependence of the muscle signal that is expected for the observed fraction of the dye (~0.1) that has changed to the calcium-bound form (Palade and Vergara, 1981; Baylor et al., 1982*b*). Qualitatively, the data points and the calibration curve have similar shapes. Both show a double-peaked increase in absorbance between 570 and 700 nm, indicating that the Arsenazo III signal in the muscle fiber is largely due to the formation of Ca^{2+}-dye complex(es). However, a clear discrepancy between the spectral shape of the muscle signal and that of the cuvette calibration is also apparent; for example, the relative amplitudes of the changes observed at 660 and 600 nm are not in agreement (see also Baylor et al., 1979, 1982*b*; Miledi et al., 1981, 1982; Palade and Vergara, 1982*b*). This finding indicates a difficulty, in that there is some difference between the Ca^{2+}-Arsenazo III reaction in myoplasm during a transient and the Ca^{2+}-dye reaction in the cuvette solution at steady state.

Fig. 2 *B* shows a similar analysis carried out for the Antipyrylazo III signal. In this case the muscle data are in extremely close agreement with the spectral shape obtained in the cuvette measurement. This finding is important, because it indicates that it may be possible to reliably calibrate the Ca^{2+}-Antipyrylazo III reaction in myoplasm using the stoichiometry, affinities, and extinction coefficients applicable to the steady-state Ca^{2+}-dye reaction in cuvette. If one uses the calibration constants given by Ríos and Schneider (1981) for the 1 Ca^{2+}:2 dye complex, and makes the additional assumption that the Ca^{2+}-dye reaction in the muscle fiber is in kinetic equilibrium with the myoplasmic free Ca^{2+} transient, the latter calibrates to a peak value of 2.2 μM for the myoplasmic increase driving the signal in Fig. 2 *B* (see Baylor et al., 1982*b*; Kovács et al., 1983).

A second difference apparent in comparing the muscle Ca^{2+}-dye signals in Fig. 1 is the earlier peak and overall faster time course for the Antipyrylazo III signal (peak at 10.4 ms following stimulation; half-width of 24 ms) compared with the Arsenazo III signal (peak at 12.8 ms; half-width of 50 ms). As shown in Table I this degree of temporal discrepancy between the two Ca^{2+}-dye signals is a rather consistent finding, not explained by differences in dye concentration or sarcomere spacing. This disparity in time courses indicates a second difficulty, because it suggests that either (*a*) one (or both) of the dyes has systematically modified the time course of the underlying free Ca^{2+} transient, or (*b*) the relationship between the free Ca^{2+} transient and the Ca^{2+}-dye signal departs from being rapid and linear for one (or both) of the dyes.

A finding consistent with the second of these possibilities is shown in Fig. 3, in which the time course of the absorbance signals as a function of wavelength is examined in more detail. The three traces superimposed at the top of either *A* or *B* (taken from the same experiments as shown

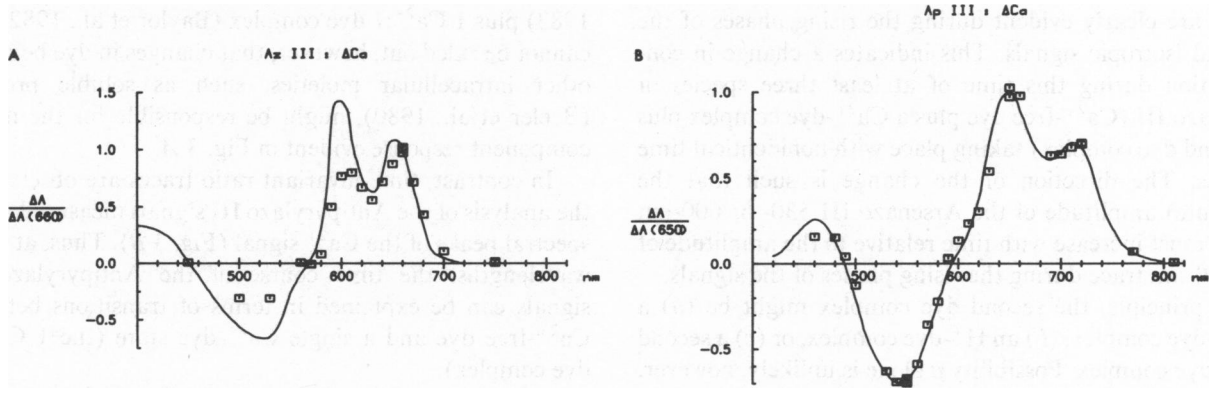

FIGURE 2 Comparison of the wavelength dependence of the amplitude of the early isotropic dye signal from a muscle fiber (□) with a scaled cuvette calibration curve (continuous line). At each wavelength the muscle data point was normalized by the amplitude of the signal that would have been observed during that sweep using light of 660 nm (in the case of Arsenazo III) or 650 nm (in the case of Antipyrylazo III), as judged from time-interpolated bracketing measurements during the run. (*A*) Arsenazo III. Calibration curve taken from Fig. 6 of Baylor et al. (1982*b*) and scaled so that the 660-nm value corresponds to 1.0. Muscle signal measured from the experiment of Fig. 1 *A*, 13.2 ms following stimulation. The data for the plot were collected over a 10-min period, during which time $A(570)$ decreased from 0.057 to 0.056, and $\Delta A(660)$ averaged 0.0031. (*B*) Antipyrylazo III. Calibration curve taken from Fig. 10 of Baylor et al. (1982*b*) and scaled so that the 650-nm value corresponds to 1.0. Muscle signal measured from the experiment of Fig. 1 *B*, 10.4 ms following stimulation. The data for the plot were collected over a 9-min period, during which time $A(550)$ varied from 0.34 to 0.29 and $\Delta A(650)$ averaged 0.0146.

TABLE I
COMPARISON OF TEMPORAL CHARACTERISTICS OF CALCIUM-DYE SIGNALS FROM ARSENAZO III AND ANTIPYRYLAZO III

Dye	Fiber ID	Sarcomere spacing	Dye concentration	Temperature	Time-to-peak	Half-width
		μm	*mM*	*°C*	*ms*	
Az III	061282.2	4.3	1.1	16.3	12.8	50.0
Az III	061282.2	4.3	0.5	16.3	12.4	46.4
Az III	070982.2	3.6	0.7	16.0	12.0	36.8
Az III	070982.2	3.7	0.5	16.0	13.6	35.9
				Mean (N = 4)	12.7	42.3
Ap III	061082.2	3.4	1.5	17.1	10.4	24.0
Ap III	061082.2	3.8	0.9	17.2	11.0	23.7
Ap III	061582.1	3.6	0.6	16.5	10.8	23.4
Ap III	070282.3	3.7	0.8	15.2	10.0	15.6
Ap III	060982.2	3.9	0.6	17.4	10.0	18.0
				Mean (N = 5)	10.4	20.9

All data were tabulated from absorbance signals measured in response to a single action potential, using 660-nm light for the Arsenazo-III injected fibers (Az III) and 650-nm light for the Antipyrylazo-III injected fibers (Ap III), and, in some cases, corrected for the small intrinsic signal observed at long wavelengths. Time-to-peak was measured from the moment of the shock and includes ~1-ms delay for propagation of the action potential to the site of optical recording. Half-width is the measured time between the signal reaching half of its peak amplitude on the rising and decaying phases.

in Figs. 1 and 2) are isotropic dye signals measured at the wavelengths corresponding to the spectral peaks of the Ca^{2+} response. If these absorbance changes reflect dye transitions between a single Ca^{2+}-free state and a single Ca^{2+}-dye state (for example, 1 Ca^{2+}:1 dye, 1 Ca^{2+}:2 dye, or 2 Ca^{2+}:2 dye, but not a mixture of these stoichiometries), the relative time courses of the signals at different wavelengths should be identical. A sensitive test for this hypothesis is to take the ratios of the signals at different wavelengths. If the ratio traces are not constant as a function of time, it must be concluded that there are dye transitions between more than two states and that these transitions take place with different time courses.

The bottom of Fig. 3 shows the results of the analysis. In the case of the Arsenazo III signals (*A*), time-varying ratio traces are clearly evident during the rising phases of the original isotropic signals. This indicates a change in concentration during this time of at least three species of Arsenazo III (Ca^{2+}-free dye plus a Ca^{2+}-dye complex plus a second dye complex) taking place with nonidentical time courses. The direction of the change is such that the (absolute) amplitude of the Arsenazo III 530- or 600-nm traces must increase with time relative to the amplitude of the 660-nm trace during the rising phases of the signals.

In principle, the second dye complex might be (*a*) a Mg^{2+}-dye complex, (*b*) an H^+-dye complex, or (*c*) a second Ca^{2+}-dye complex. Possibility *a* alone is unlikely, however, as the direction of the ratio changes is opposite to that expected for the dissociation of resting Mg^{2+}-dye to free dye in response to the formation of Ca^{2+}-dye complex (Baylor et al., 1982*b*). Possibility (*b*) alone is more difficult to evaluate in the absence of kinetic information concerning the speed of the possible H^+-dye reaction(s). However, if the reaction(s) is not kinetically limiting, the required amplitude of the H^+-dye effect is about an order of magnitude larger than that expected on the basis of the small alkalization of myoplasm detected in fibers injected with the H^+-indicator dye phenol red (Baylor et al., 1982*a*), although its sign is in the right direction (Baylor et al., 1982*b*). On the other hand, possibility (*c*) might be considered likely, as it would be consistent with the multicomponent stoichiometry that has been observed for the calcium-Arsenazo III reaction in steady-state cuvette measurements (Thomas, 1979; Ogawa et al., 1980; Palade and Vergara, 1981, 1982*a*, 1983; Ríos and Schneider, 1981; Baylor et al., 1982*b*). For example, the direction of the ratio change appears to be consistent with the idea that the proportion of 2 Ca^{2+}:2 dye complex (Palade and Vergara, 1982*a*, 1983) increases with time relative to the proportion of 1 Ca^{2+}:2 dye complex (Palade and Vergara, 1982*a*, 1983) plus 1 Ca^{2+}:1 dye complex (Baylor et al., 1982*b*). It cannot be ruled out, however, that changes in dye bound to other intracellular moieties, such as soluble proteins (Beeler et al., 1980), might be responsible for the multicomponent response evident in Fig. 3 *A*.

In contrast, time-invariant ratio traces are observed in the analysis of the Antipyrylazo III signals measured at the spectral peaks of the Ca^{2+} signal (Fig. 3 *B*). Thus, at these wavelengths, the time course of the Antipyrylazo III signals can be explained in terms of transitions between Ca^{2+}-free dye and a single Ca^{2+}-dye state (the 1 Ca^{2+}:2 dye complex).

It may be concluded that the isotropic Ca^{2+}-dye signal from Antipyrylazo III in myoplasm has three favorable properties in comparison with the signal from Arsenazo III: (*a*) its time course is significantly faster, which suggests it provides a temporally more accurate monitor of the underlying free Ca^{2+} transient; (*b*) its spectral shape superimposes that of a Ca^{2+}-dye difference spectrum obtained in cuvette, which raises the likelihood that a

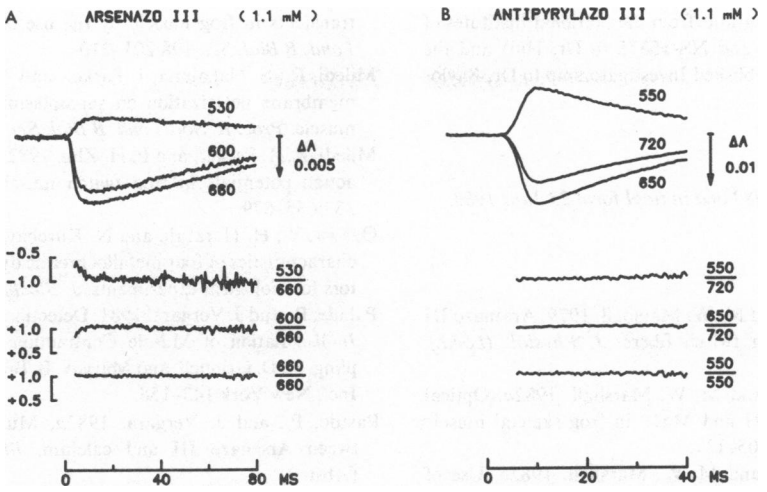

FIGURE 3 Ratio analysis testing the hypothesis that the isotropic dye signals measured at different wavelengths have identical time courses. A time-varying ratio trace indicates failure of the hypothesis (see text for additional details). Same experiments as in Figs. 1 and 2. The ratio traces (*lower*) are not shown at times earlier than about halfway in the rising phases of the isotropic signals (*upper*); this corresponds to the earliest time that the ratio traces become well defined. Prior to this they break up, as their denominators tend towards small values with relatively large fluctuations. (*A*) Arsenazo III. *Upper* three traces (superimposed) are single-sweep examples of dye-related isotropic signals. The wavelength (in nanometers) is indicated next to each trace. No correction was made in these records for intrinsic absorbance changes measured at 750 nm, since, as shown in Fig. 1 *A*, they were negligible for this fiber. The fourth trace is the ratio of a single 530-nm trace to the average of two bracketing 660-nm traces; this ratio trace has been scaled by the factor 1/0.312, to make its average value at later times equal to unity. The fifth trace is the ratio of a single 600-nm trace to the average of two bracketing 660-nm traces. This ratio trace has been scaled by the factor 1/0.763. The lowermost trace is the ratio of a single 660-nm trace to the average of two bracketing 660-nm traces (taken at the beginning and end of the entire run); this trace has been scaled up by the factor 1/0.974. It represents a control for the method and should be time-invariant if the fiber condition was stable during the run. (*B*) Antipyrylazo III. *Upper* three traces (superimposed) are single-sweep examples of dye-related isotropic signals. A small correction has been made in these signals for the non–dye-related change seen in the 810-nm traces (Fig. 1 *B*); namely, the latter signal was scaled up by the factor $(810/\lambda)^2$ and subtracted from each of the signals recorded at wavelength λ. The fourth trace is the ratio of a single 550-nm trace to a single 720-nm trace taken 0.5 min later; this ratio trace has been scaled up by the factor 1/0.998. The fifth trace is the ratio of a single 650-nm trace to the same single 720-nm trace, taken 0.2 min later; this trace has been scaled by the factor 1/1.469. The *lowermost* trace is the ratio of a single 550-nm signal (the same as that used in computing the 550 to 720 nm ratio trace, to the average of two bracketing 550-nm traces (taken 2.6 min earlier and 2.1 min later, at the beginning and end of the run); this trace has been scaled by the factor 1/1.018. As in *A*, the *lowermost* trace represents a control for fiber instability. The calibration bars in the *lower* half of *A* also apply to the *lower* half of *B*.

reliable in vivo calibration of its amplitude may be obtained from in vitro measurements; and (*c*) as judged by the ratio analysis in Fig. 3 *B*, its waveform at different wavelengths is consistent with a single Ca^{2+}-dye stoichiometry. By these criteria then, the Ca^{2+}-Antipyrylazo III signal appears to be simpler and more easily interpreted than the Ca^{2+}-Arsenazo III signal. Nevertheless, the use of Antipyrylazo III as a myoplasmic Ca^{2+} indicator is not without significant complexity of its own, as evidenced by the early dichroic and late isotropic components of the signals in Fig. 1 *B* (see also Baylor et al., 1982b; Quinta-Ferreira et al., 1983). The characteristics of these components will be described in further detail elsewhere.

Finally, note that experiments of this general type, comparing signals from Arsenazo III and Antipyrylazo III in frog muscle, have been carried out previously in the cut fiber preparation (Palade and Vergara, 1982*b*), although in that case two separate methods were used for introducing the dyes into myoplasm (Antipyrylazo III diffused in from the cut end, whereas Arsenazo III was ionophoresed locally through a micro-electrode). The results of the dye comparisons in cut fibers in response to a single action potential at 21°C show some similarities as well as some differences compared with the results at 16°C described here. For example, nonidentical time courses of the Arsenazo III signals recorded at 530, 600, and 660 nm were also detected (Fig. 5 of Palade and Vergara, 1982*b*). However, the spectral dependence of the peak of the Antipyrylazo III signal (see Fig. 8 of Palade and Vergara, 1982*b*) appears to be in significant disagreement with the cuvette calibration shown in Fig. 2 *B* above. Moreover, the Antipyrylazo III 550-nm signal at late times had a time course clearly different from that of the 650-and 710-nm signals (also Fig. 8, Palade and Vergara, 1982*b*). In addition, there appears to be less discrepancy in overall time course between the Arsenazo III 660-nm signal and the Antipyrylazo III 650-nm signal in cut fibers at 21°C than in intact fibers at 16°C (see Table IV, Palade and Vergara, 1982*b*). The exact basis for the differences in results between the two sets of experiments is not understood, but is unlikely to be due simply to the temperature difference.

Financial support was provided by grants from the National Institutes of Health (NS-17620 to Dr. Baylor and NS-15375 to Dr. Hui) and the American Heart Association (Established Investigatorship to Dr. Baylor and research grant to Dr. Hui).

Received for publication 5 May 1983 and in final form 23 June 1983.

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