Ca^{2+} BINDING KINETICS OF FURA-2 AND AZO-1 FROM TEMPERATURE-JUMP RELAXATION MEASUREMENTS

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ABSTRACT The Ca^{2+}-binding kinetics of fura-2 and azo-1 were studied using temperature-jump relaxation methods. In 140 mM KCl at 20°C, the association and dissociation rate constants for fura-2 were 6.02×10^8 $M^{-1}s^{-1}$ and 96.7 s^{-1}, respectively. The fura-2 kinetics were insensitive to pH over the range 7.4 to 8.4. Azo-1 was studied in 140 mM KCl, at pH 7.4, at 10° and 20°C. At 10°C, azo-1 exhibited association and dissociation rate constants of 1.43×10^8 $M^{-1}s^{-1}$ and 777.9 s^{-1}, respectively; while at 20°C, the corresponding values were 3.99×10^8 $M^{-1}s^{-1}$ and 1,177 s^{-1}. The kinetic results demonstrate that fura-2 and azo-1 are well suited to monitoring rapid changes in intracellular $[Ca^{2+}]$.

INTRODUCTION

Fura-2 and azo-1 are Ca^{2+} indicators which have been used extensively to measure the changes in intracellular Ca^{2+} concentration in a wide variety of biological systems. The equilibrium properties of these indicators have been described elsewhere (Grynkiewicz et al., 1985; Tsien, 1983). If an indicator is to reflect accurately the instantaneous changes in Ca^{2+} concentration, then the kinetics of the reaction between the indicator and Ca^{2+} must be significantly faster than the rate at which the Ca^{2+} concentration is changing. Since fura-2 and azo-1 are used in systems such as muscle and nerve where the intracellular Ca^{2+} concentration can change quite rapidly, it is important to know precisely the kinetics of the Ca^{2+}-binding reactions of these indicators. Here we have investigated the kinetics of the Ca^{2+}-binding reactions of fura-2 and azo-1 by the temperature-jump relaxation technique. In this classic technique (Eigen and DeMayer, 1963), a sample of indicator partially saturated with Ca^{2+} is heated within a few microseconds by discharging a capacitor through it. The sudden rise in temperature shifts the equilibrium between free and Ca^{2+}-bound indicators. The time needed to re-attain equilibrium reflects the rates of Ca^{2+} association and dissociation. Because no physical mixing is required, the temperature-jump technique can measure reactions too fast for stopped-flow methods.

MATERIALS AND METHODS

Samples used for the fura-2 measurements consisted of 5.33 μM fura-2 in a solution containing 140 mM KCl, 30 mM Tris/TrisHCl[tris-(hydroxymethyl)aminomethane], 30 mM K_2H.HEEDTA [N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid] and 4.53-25.0 mM $CaCl_2$, at pH 7.40 and 8.40. For the azo-1 experiments, the samples contained 10.4 μM azo-1, 140 mM KCl, 30 mM Tris/TrisHCl, 30 mM K_2H.HEEDTA, and 5.0–22.7 mM $CaCl_2$ at pH 7.40. Each sample solution was mixed and then kept in a temperature bath held at 10.0° or 20.0°C and the pH was adjusted to the desired value at the set temperature. Each solution was then split into two aliquots for duplicate samples. Up to 12 temperature-jump measurements were taken on each aliquot.

The temperature-jump kinetic spectrometer used was manufactured by Dialog (Garching, W. Germany) and has been described in detail previously (Rigler et al., 1974). For measurements on fura-2, the spectrometer was operated with fluorescence detection. Light from a 200 W Xe-Hg arc lamp was passed through a monochromator to produce 380-nm light with a bandwidth of 6.6 nm. The 380-nm light was used to excite the sample and the fura-2 fluorescence was passed through a 390 nm long-pass filter and collected in a photomultiplier tube positioned at 90° to the path of the exciting beam. The azo-1 measurements were performed with absorbance detection on the instrument. Light at 475 nm with 6.6 nm bandwidth was used to probe azo-1. A 100 W W-halogen lamp served as the light source. Light transmitted through the sample was collected in a photomultiplier tube.

Before measurement, each sample was filtered centrifugally through a membrane filter of 0.45 μm porosity. The sample was then purged by bubbling water-saturated helium through the solution for 3–5 min to remove dissolved gasses which may form bubbles inside the sample cell when the solution is abruptly heated by the temperature jump. Helium is used because in the temperature range of these experiments, the solubility of helium in water increases with increasing temperature. The initial temperature of the thermally equilibrated sample (2.4° or 12.4°C) was monitored to within 0.2°C using a 2100A digital thermometer (John Fluke Mfg. Co., Inc., Seattle, WA) fitted with a copper-constantan thermocouple in contact with the upper electrode of the sample cell. To effect the temperature jump, a 0.05 μf capacitor charged to 25 kV was discharged through the sample. This yielded a temperature jump of 7.6°C, resulting in a final temperature of 10° or 20°C. Each relaxation curve was digitized as 2,048 time points on a 805 transient recorder (Biomation Cupertino, CA). The digitized data were subsequently transferred via a PET microcomputer (Commodore Business Machines, Santa Clara, CA) to a VAX 11/780 minicomputer where all data reduction and analyses were performed. The program DISCRETE (Provencher, 1976*a*, *b*) was used to analyze the relaxation data for exponential relaxation times.

RESULTS

The Ca^{2+}-binding reaction of an indicator like fura-2 or azo-1 should be simply described as

$$In^{n-} + Ca^{2+} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} InCa^{(n-2)-},$$

Scheme I

where In^{n-} is the indicator anion; $InCa^{(n-2)-}$, the Ca^{2+}-bound form of the indicator and k_a and k_d are the association and dissociation rate constants, respectively. If In^{n-} is the only Ca^{2+}-binding species in the solution, then the relaxation time, τ, is given by

$$\frac{1}{\tau} = k_a([In^{n-}] + [Ca^{2+}]) + k_d, \quad (1)$$

where $[In^{n-}]$ and $[Ca^{2+}]$ refer to the equilibrium concentrations of free In^{n-} and free Ca^{2+} at the final sample temperature after the temperature jump has occurred. The simplest experimental approach would have been to prepare the sample solution with equal total concentrations of Ca^{2+} and In^{n-} ($C^0_{Ca^{2+}} = C^0_{In^{n-}}$). In such a case, we may use equilibrium conditions to rearrange Eq. (1) to give (see e.g., Bernasconi, 1976)

$$\frac{1}{\tau^2} = 4k_ak_dC^0_{Ca^{2+}} + k_d^2. \quad (2)$$

We attempted this experiment and found that in order to get experimentally accessible relaxation times, the concentrations of the reactants must be reduced to a level at which contamination by extraneous Ca^{2+} became severe even when ultrapure KCl was used as a background electrolyte.

The alternative approach we eventually adopted was to conduct the measurement using a solution wherein both the Ca^{2+} and pH were heavily buffered. When the buffer concentrations are much greater than the concentration of the indicator, the relaxation time for reaction scheme I becomes

$$\frac{1}{\tau} = k_a[Ca^{2+}] + k_d, \quad (3)$$

where $[Ca^{2+}]$ is the concentration of free Ca^{2+} as determined by the HEEDTA buffer equilibrium at the final temperature of the sample. For Eq. (3) to be valid, the rate at which the buffer systems attain equilibrium at the final temperature must be significantly faster than the rate at which an indicator relaxes to its new equilibrium. We tested this condition by varying the concentrations of the pH and the HEEDTA buffer concentrations independently in different control samples. Reducing the Tris buffer concentration to one third of that actually used in the measurements produced no significant changes in the measured relaxation times. Similarly, doubling the HEEDTA concentration yielded relaxation times which were essentially unchanged. We are confident, therefore, that the relaxation times obtained in these experiments accurately reflect the kinetics of the indicators themselves.

In these experiments we have taken advantage of the fact that the pH of the Tris buffer system is strongly dependent on temperature. Immediately after the temperature jump, the Tris buffer attains a lower pH. The drop in pH reduces the Ca-binding affinity of HEEDTA and causes the concentration of free Ca^{2+} in the solution to be buffered at a higher level. The indicators then adjust to the new, higher $[Ca^{2+}]$ by binding Ca^{2+}. It is this change in the amount of Ca-bound indicator that was monitored spectrophotometrically in the experimental apparatus. In the case of azo-1, binding of Ca^{2+} causes a decrease in the absorbance of the indicator at 475 nm. Since transmitted light was actually monitored, an increase in light level was observed. The rising experimental trace in Fig. 1 illustrates this. It is also evident from Fig. 1 that the chemical relaxation for azo-1 occurs more quickly at 20° then at 10°C, as one would expect for simple kinetics. These curves were well fit by a single exponential. When excited by light at 380 nm, fura-2 shows diminished fluorescence emission upon binding Ca^{2+}. In confirmation of this, Fig. 2 shows the relaxation curve of fura-2 to be an exponential decay. An additional feature of the fura-2 experimental trace is that immediately after the temperature jump at $t = 0$, the fluorescence drops precipitously. ~20% of the total change in amplitude corresponds to this initial drop. This very rapid decrease in fluorescence is not due to chemical relaxation but is rather the result of physical processes which reduce the efficiency of fluorescence emission in the

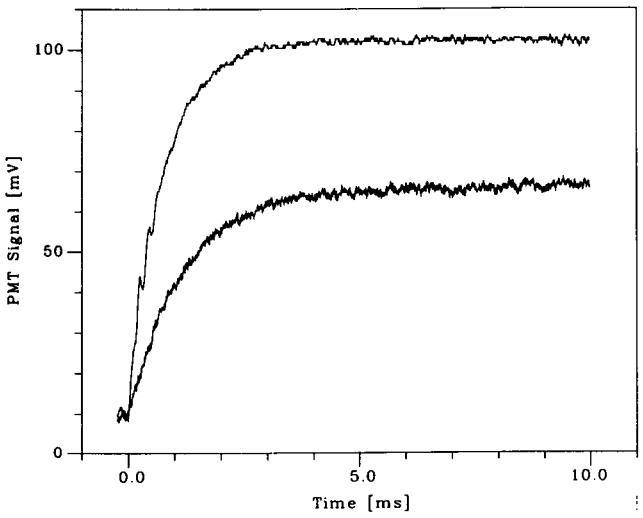

FIGURE 1 Relaxation curves for azo-1 at 10.0°C (*lower trace*) and 20.0°C (*upper trace*). The trace at 10.0°C was under conditions where $[Ca^{2+}] = 0.335$ μM. The 20.0°C trace was taken on a sample wherein $[Ca^{2+}] = 0.314$ μM. The two samples were of identical composition: 10.4 μM azo-1, 140 mM KCl, 30 mM Tris, 30 mM HEEDTA, and 5.0 mM $CaCl_2$ at pH = 7.40. In recording the photomultiplier signal, an arbitrary DC voltage offset was applied. The vertical scale is not representative of absolute signal strength.

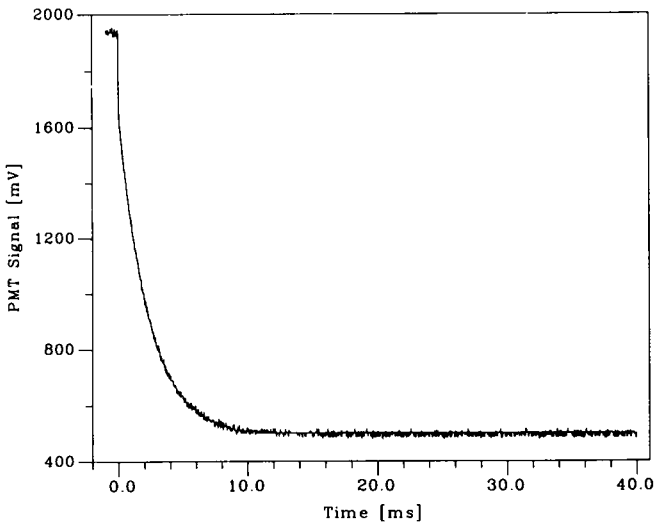

FIGURE 2 Relaxation curve for fura-2 at 20.0°C. The measurement was made on a sample which contained 5.3 μM fura-2, 140 mM KCl, 30 mM Tris, 30 mM HEEDTA and 7.90 mM $CaCl_2$, at pH = 7.40. Under these conditions, $[Ca^{2+}]$ = 0.561 μM. An arbitrary DC voltage offset was applied when recording the photomultiplier signal. The vertical scale does not correspond to absolute signal strength.

fura-2 molecules with increasing temperature. These physical processes are extremely fast. Therefore, on the time scale of the chemical relaxation, the fluorescence decrease accompanying the physical processes appear "instantaneous." The portion of the experimental trace corresponding to chemical relaxation was well fit by a single exponential.

From each relaxation curve a relaxation time was extracted. The relaxation times were then plotted as a function of the buffered Ca^{2+} concentrations, which were calculated using published thermodynamic data for HEEDTA (Martell and Smith, 1974). A linear least squares fit of the plot to Eq. (3) enabled us to determine k_a and k_d. Linear fits to the fura-2 and azo-1 data are presented in Figs. 3 and 4, respectively. Each point in the plots is the average of from 8 to 20 replicate measurements. In Fig. 3 we present the fura-2 data at pH 7.40 as well as pH 8.40. Since the most basic proton on fura-2 has a $pK_a \approx 6.4$, we do not expect the kinetic behavior of fura-2 to be dependent on pH above pH 7.40. Indeed, it is quite apparent from Fig. 3 that fura-2 exhibits the same kinetics at pH 8.40 as at pH 7.40. We have therefore combined the data sets at pH 7.40 and 8.40 in the linear least squares analysis to obtain k_a and k_d for fura-2.

Rate constants derived from the least squares fits for fura-2 and azo-1 are listed in Table I. Dissociation constants (K_d's) derived from kinetic and equilibrium measurements are also given in Table I for comparison. Since we investigated azo-1 at two temperatures, it was possible to make estimates of the kinetic activation parameters for azo-1. These activation parameters as well as some derived thermodynamic parameters for azo-1 are tabulated in Table II.

DISCUSSION

We first direct our attention to the process by which the Ca^{2+}-indicator complex is formed. Complex formation is accomplished by a collisional encounter between a Ca^{2+} and an indicator molecule and subsequent attachment of the coordinating groups of the indicator to the Ca^{2+}. In the present study, both fura-2 and azo-1 bind Ca^{2+} and the chelating functional groups are essentially identical for the two indicators, therefore we do not expect to see much

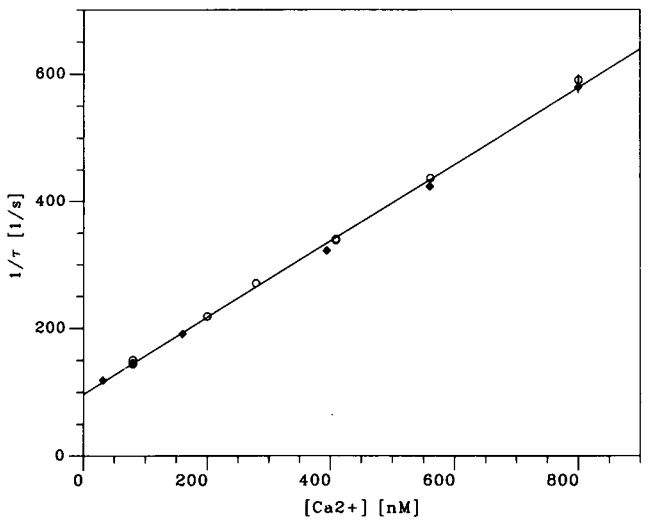

FIGURE 3 Plot of inverse relaxation time vs. $[Ca^{2+}]$ for fura-2 at 20.0°C. Data taken at pH 7.40 are represented by O. Data collected at pH 8.40 are plotted as ♦. Standard error within a set of measurements comprising a data point is plotted as an error bar through the symbol. Where not shown, the error bars are smaller than or equal to the dimension of the plot symbol. The line shown is the linear least squares fit of all the data to Eq. 3.

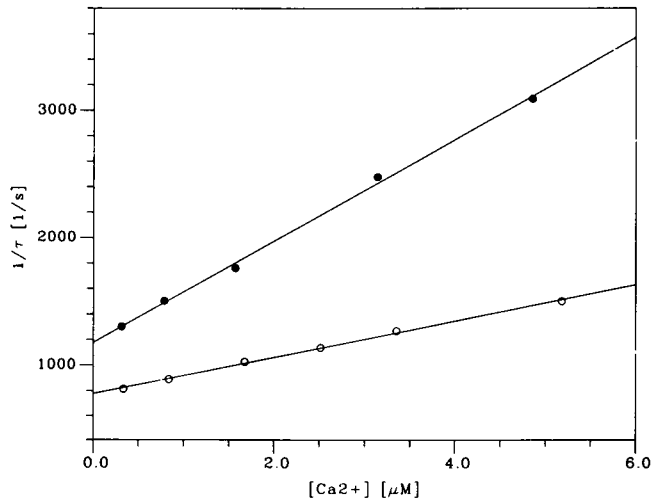

FIGURE 4 Plots of inverse relaxation time vs. $[Ca^{2+}]$ for azo-1 at 10.0°C (O) and 20.0°C (●). The lines shown are the linear least squares fits of the data to Eq. 3.

TABLE I
ASSOCIATION AND DISSOCIATION CONSTANTS FOR THE Ca^{2+}-BINDING REACTIONS OF FURA-2 AND AZO-1

	T	pH	k_a	k_d	$K_{d,kin}$*	$K_{d,therm}$
	°C		$M^{-1}s^{-1}$	s^{-1}	μM	μM
Fura-2	20	7.4 and 8.4	$6.02 (\pm 0.07) \times 10^8$	96.7 ± 2.9	0.161	0.135‡
Azo-1	20	7.4	$3.99 (\pm 0.11) \times 10^8$	1177 ± 29	2.95	2.63§
	10	7.4	$1.43 (\pm 0.03) \times 10^8$	777.9 ± 9.6	5.45	—

*$K_{d,kin} = k_d/k_a$.
‡Determined in 100 mM KCl, 10 mM K-MOPS, pH 7.1–7.2, 20°C.
§Determined in 100 mM KCl, 10 mM HEPES, pH 7.4, 19.5°C.

difference between the association rate constants for the two molecules. Examining Table I shows this to be true as fura-2 and azo-1 at 20°C exhibit k_a's of 6.02×10^8 $M^{-1}s^{-1}$ and 3.99×10^8 $M^{-1}s^{-1}$ respectively. Since the association rate constants are similar to each other and not much different from what is expected for the process of complex formation where water loss from the inner coordination sphere of the Ca^{2+} is generally rate-limiting (Hague, 1977), the difference in affinities of the two indicators for Ca^{2+} must be reflected in the dissociation rate constants. Indeed, azo-1, the weaker Ca^{2+} binder, has a k_d that is more than an order of magnitude larger than that of fura-2.

From Table I, we can see that the azo-1 rate constants fit the usual pattern of higher rate with increasing temperature. A little more is revealed by the contents of Table II. $\Delta H°_{assoc}$ for binding Ca^{2+} to azo-1 is +10 kcal/mol, so the association reaction is endothermic. However, at a $\Delta S°_{assoc}$ of 60 cal/mol.K, binding is entropically favored. At 20°C, $-T\Delta S°_{assoc}$ is −18 kcal/mol, which more than compensates for the unfavorable $\Delta H°_{assoc}$.

The linear dependence of the inverse relaxation times on free $[Ca^{2+}]$ (Fig. 3 and 4) supports the simplest possible kinetic scheme as described by scheme I and Eq. 3. This simplicity is one advantage of these indicators and related tetracarboxylate chelators over earlier metallochromic dyes such as arsenazo III and antipyrylazo III. Tested under comparable temperature-jump measurements, arsenazo III shows much more complicated kinetics (Dorogi et al., 1983; J. P. Y. Kao, unpublished results) than described here, perhaps because of its complicated stoichiometry (see, for example, Palade and Vergara, 1983).

TABLE II
ACTIVATION AND THERMODYNAMIC PARAMETERS FOR THE AZO-1 Ca^{2+}-BINDING REACTION

	E_a*	$\Delta S^{\ddagger}$*	$\Delta H°_{kin}{}^{\ddagger}$	$\Delta S°_{kin}{}^{\ddagger}$
	kcal/mol	cal/mol · K	kcal/mol	cal/mol · K
Association	17	37	10	60
Dissociation	6.8	−23	−10	−60

*Activation energy E_a is calculated using the expression $d\ln k_i/d(1/T) = -E_a/R$ and the entropy of activation $\Delta S^{\ddagger}$ was calculated using the expression $k_i = (kT/h) \exp[-(E_a - RT)/RT] \exp[\Delta S^{\ddagger}/R]$, where k_i is the rate constant (k_a or k_d) determined at the absolute temperature T, h is the Planck constant and R is the gas constant (see e.g., Moore, 1972).
‡For association, $\Delta H°_{kin} = E_{a,assoc} - E_{a,diss}$ and $\Delta S°_{kin} = \Delta S^{\ddagger}_{assoc} - \Delta S^{\ddagger}_{diss}$. Corresponding values for the dissociation process are the negatives of those for association. The subscript *kin* is used to indicate that the thermodynamic quantities are derived from kinetic rather than equilibrium measurements.

Comparison of the dissociation constants derived kinetically with those obtained by standard measurements shows that the agreement is rather good. In the case of fura-2, the kinetic value is ~16% higher than the thermodynamic value while for azo-1, the kinetic value is ~11% higher. Since the kinetic measurements are more difficult and indirect than the equilibrium measurements, we consider the agreement satisfactory, though the dissociation constants derived from equilibrium titrations are to be preferred.

Ashley and colleagues have very recently published independent stopped-flow measurements of the rate of Ca^{2+} dissociation from fura-2 and indo-1 (Jackson et al., 1987). Their value of k_d for fura-2, 85 s^{-1}, obtained by mixing Ca^{2+}-saturated dye with excess EGTA or EDTA, is in reasonable agreement with ours. The stopped-flow experiments suffered from the disadvantage of not being able to provide an independent determination of the association rate constant. In contrast, as described in the Results section, a temperature-jump experiment yields simultaneously independent values for both the association and dissociation rate constants for fura-2 and azo-1. Moreover, since stopped-flow has a much longer dead time than temperature-jump, it would have been unable to resolve the faster kinetics of azo-1.

We now address the question of the suitability of these indicators for measuring rapid changes in Ca^{2+} concentration. The relevant parameter to consider here is the relaxation time under the conditions of measurement. Two limiting cases are of interest. In the first, we assume that the indicator is used at a low enough concentration to probe the free Ca^{2+} concentration in the cell without significantly buffering it. Under these conditions, the proper expression for the relaxation time is Eq. (3). Given its dependence on $[Ca^{2+}]$ (Fig. 3 and 4), no single response time can be given. But at, say, 1 μM $[Ca^{2+}]$, as might be achieved during a transient, Eq. (3) yields 1.4 and 0.63 ms for the relaxation times of fura-2 and azo-1 respectively, at 20°C. We can

expect an indicator to track reasonably accurately any Ca^{2+} concentration change that occurs on a time scale 3 to 4 times slower than its relaxation time. At 20°C, fura-2 should be able to track transients that rise or fall on the order of 5 ms or slower. Azo-1, on the other hand, could be used to measure transients that have rise or fall times on the order of 2 ms or slower. The response time naturally improves at higher temperatures since all the relevant rate constants increase with temperature. Using the activation energies given in Table II, we can calculate for azo-1 at 37°C $k_a = 1.97 \times 10^9\ M^{-1}s^{-1}$ and $k_d = 2{,}239\ s^{-1}$. Substituting these values into Eq. (3) leads to a relaxation time of 0.24 ms at 37°C; a 2.7-fold improvement over the response time at 20°C. Since the two indicators are similar, we expect roughly the same improvement for fura-2 when used at 37°C. We emphasize again that the numerical estimates given above apply only when $[Ca^{2+}]$ is $\sim 1\ \mu M$. The response time will be relatively longer at lower free Ca^{2+} concentrations and shorter at higher free Ca^{2+} concentrations.

The other limiting case is when a large excess of indicator is used so that it becomes the dominant buffer. Here the changing Ca^{2+}-occupancy of the dye reflects the total flux of Ca^{2+} into or out of the compartment containing the dye, rather than the free $[Ca^{2+}]$ change that would have occurred in the absence of indicator. Now the relevant kinetics approximate Eq. (1). Because millimolar indicator concentrations are generally needed to provide buffering sufficient to overwhelm that of the cell, relaxation times of just a few microseconds are predicted, fast enough for nearly any biological problem.

The above calculations assume the *in vitro* kinetics measured in this work. Of course, the possibility exists that inside cells the dyes may behave somewhat differently, for example due to the viscosity or tortuosity of the cytoplasm, or even the presence of dye-binding constituents. Indeed, experiments on skeletal muscle (Baylor et al., 1985; Hollingsworth and Baylor, 1987) can be interpreted to suggest that azo-1 and fura-2 have slower kinetics than would be predicted by the data reported here and that both azo-1 and fura-2 respond more slowly than antipyrylazo III to Ca^{2+} transients in skeletal muscle. It is not clear to us how much of the apparent slowing is due to viscosity, dye binding, or possible spatial microheterogeneity of $[Ca^{2+}]$ during muscle contraction. The studies of Vergara and Delay, however, revealed no substantial difference in the responses of azo-1 and antipyrylazo III to Ca^{2+} transients in skeletal muscle fibers (Vergara and Delay, 1986). It should be pointed out that the work of Vergara and Delay focussed on the rising phase of the muscle Ca^{2+} transient whereas in the studies of Baylor and colleagues the apparently slow response of fura-2 and azo-1 was manifest primarily in the falling phase of the Ca^{2+} transient. It seems clear that much work is needed before the *in vivo* behavior of these Ca^{2+}-sensitive dyes can be fully understood. In any case, good *in vitro* measurements are necessary as a baseline for further investigation.

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