Effects of Temperature on Calcium-Sensitive Fluorescent Probes

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ABSTRACT The effect of temperature on the binding equilibria of calcium-sensing dyes has been extensively studied, but there are also important temperature-related changes in the photophysics of the dyes that have been largely ignored. We conducted a systematic study of thermal effects on five calcium-sensing dyes under calcium-saturated and calcium-free conditions. Quin-2, chlortetracycline, calcium green dextran, Indo-1, and Fura-2 all show temperature-dependent effects on fluorescence in all or part of the range tested (5–40°C). Specifically, the intensity of the single-wavelength dyes increased at low temperature. The ratiometric dyes, because of variable effects at the two wavelengths, showed, in general, a reduction in the fluorescence ratio as temperature decreased. Changes in viscosity, pH, oxygen quenching, or fluorescence maxima could not fully explain the effects of temperature on fluorescence. The excited-state lifetimes of the dyes were determined, in both the presence and absence of calcium, using multifrequency phase-modulation fluorimetry. In most cases, low temperature led to prolonged fluorescence lifetimes. The increase in lifetimes at reduced temperature is probably largely responsible for the effects of temperature on the physical properties of the calcium-sensing dyes. Clearly, these temperature effects can influence reported calcium concentrations and must therefore be taken into consideration during any investigation involving variable temperatures.

INTRODUCTION

Fluorescent analogues of calcium chelators have proven extremely useful in measuring the intracellular calcium concentration of various types of living cells. Fluorescent probes that have been widely used include Indo-1 (June and Rabinovitch, 1994; Sipido and Callewaert, 1995; Jacquemond and Allard, 1998; Sebille et al., 1998; Ju and Allen, 1998; Grynkiewicz et al., 1985), Fura-2 (Xu et al., 1997; Sipido and Callewaert, 1995; Chen et al., 1996; Zicha et al., 1996; Greco et al., 1996; Bhattacharya and Chakrabarti, 1998; Miao et al., 1998; Grynkiewicz et al., 1985), Quin-2 (Tsien and Pozzan, 1989; Deber and Hsu, 1986; Jacob et al., 1987; Nijayaraghavan and Hoskins, 1989), calcium green dextran (Zimprich and Bolsover, 1996; Harris, 1994; McClellan et al., 1994; Tamm and Terasaki, 1994; Kono et al., 1996; Bi et al., 1995; Carroll et al., 1994; Fetcho and O'Malley, 1995), and chlortetracycline (Tao et al., 1992; Jy and Haynes, 1984, 1987, 1988). Most experiments of this type are performed at constant temperature (e.g., 37°C). However, if temperature is varied during the investigation, the effect of that temperature change on the fluorescent probe itself must be considered during data interpretation.

One important effect of temperature on the fluorescent calcium-sensing dyes is a variation in the binding characteristics of the chelator. As has been documented for Indo-1, Fura-2 (Shuttleworth and Thompson, 1991; Howarth et al., 1995), Fluo-3 (Lattanzio, 1990), and the calcium chelators EGTA and BAPTA (Harrison and Bers, 1987), the binding equilibria are shifted such that the dissociation constant (K_d) increases at low temperature. Fortunately, this type of error is easily avoided by using the appropriate temperature-corrected K_d for a given dye (Shuttleworth and Thompson, 1991). The calcium affinities of chelators under various conditions can be measured using Scatchard plot analysis, or calculated using the van't Hoff isochore (Harrison and Bers, 1987, 1989). Further, computer programs, such as MaxChelator (Bers et al., 1994), can be used to estimate K_d at various values of temperature and ionic strength. Because this type of error has been thoroughly investigated, it will not be considered in the current study.

A second important consideration when temperature is varied during the course of an experiment is the fact that the fluorescence itself is affected by temperature, regardless of binding characteristics. In general, quantum yield (Eastman and Rosa, 1968; Song et al., 1975; Cornelissen-Gude and Rettig, 1998; Haynes et al., 1993), and thus fluorescence intensity (Connors et al., 1998; Park, 1996; Law, 1994) both increase as temperature decreases, and, in polar solvents, the effect is even more pronounced (Waris et al., 1988; Bark and Force, 1993). In addition, excited-state lifetimes of fluorescent molecules are strongly affected by temperature, showing, in most cases, longer lifetimes at lower temperatures (Drain et al., 1998; Cornelissen-Gude and Rettig, 1998; Kumke et al., 1997; Young et al., 1997; van den Zegel et al., 1984). This effect of increasing fluorescence lifetimes is largely due to a decrease in the rates of nonradiative decay at low temperature (Lee and Robinson, 1984; Lam et al., 1998; Young et al., 1997; Giri, 1992). Exceptions to the above trends have been noted, however. For instance, certain tryptophan dipeptides show an increase in quantum yield and intensity at elevated temperatures, due to an

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equilibrium shift toward an unprotonated species with a larger quantum yield (Brancaleon et al., 1995, 1997). Thus, temperature effects on fluorescence often cannot be predicted, and must be measured.

These two distinct consequences of temperature on the calcium-sensitive dyes (i.e., effects on binding characteristics and effects on fluorescence) are both crucial to the accurate and reliable use of these probes for measuring free calcium concentrations, yet frequently, neither are considered. Some groups avoid the problem of the K_d effects by reporting the $\lambda 1/\lambda 2$ ratio instead of calcium concentration (Kenyon and Goff, 1998; Gambassi et al., 1994). This is the ratio of fluorescence intensities (I) for calcium-bound to calcium-free fluorophore (I_{+Ca}/I_{-Ca}). This method, however, still does not control for the effects of temperature on the fluorescence intensity at those two wavelengths. In fact, although consideration of the temperature effects on K_d is fairly common (Puglisi et al., 1996; Liu et al., 1991; Shuttleworth and Thompson, 1991), our literature search revealed only one paper in which the effects of temperature on fluorescence were considered (Zhao and Buhr, 1995).

In the current study, thermal effects on five calcium-sensitive dyes were studied in the range of 5 to 40°C. All the dyes tested were affected by temperature to some extent. Because fluorescence can be strongly affected by certain physical characteristics of the system, including pH (Martin and Jain, 1994; Ritucci et al., 1996; Rink et al., 1982b; Szmacinski and Lakowicz, 1993), viscosity (Swaminathan et al., 1997; Somogyi et al., 1994; Luby-Phelps et al., 1993; Busa, 1992), and the presence of oxygen (Damdinsuren et al., 1995; Danielsen et al., 1995; Lakowicz, 1983), each of these, as well as the influence of temperature on the excited-state lifetimes, was studied as a possible cause for the fluorescence changes with temperature. Regardless of the causal mechanism, however, this study shows that the effects of temperature on fluorescence, as well as on the binding characteristics of the chelators, must be considered to obtain meaningful data with calcium-sensitive fluorescent dyes when temperature is varied during the course of the investigation.

MATERIALS AND METHODS

Measuring fluorescence

Fura-2, Indo-1, Quin-2, and calcium green dextran were obtained from Molecular Probes, Inc. (Eugene, OR), and chlortetracycline (CTC) was obtained from CalBiochem (La Jolla, CA). The fluorescence intensities of the probes were measured at the following concentrations: 4 μM Fura-2 pentapotassium salt; 1 μM Indo-1 pentapotassium salt; 37 μM Quin-2; 75 μM CTC HCl; 0.5 μM calcium green-1 dextran potassium salt (MW 10,000). Probes were dissolved in phosphate buffered saline (PBS) (9.4 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 100 mM NaCl, pH 7.2) at 20°C. Samples measured in the absence of calcium also contained 10 mM ethylene glycol-bis-(b-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and samples containing calcium were adjusted to 1 mM $CaCl_2$ with Tyrodes wash (148 mM NaCl, 2.5 mM KCl, 23 μM $MgCl_2$, 5 mM glucose) containing 10 mM $CaCl_2$.

Static fluorescence was measured with a Hitachi F-2000 fluorescence spectrometer controlled by a PC equipped with the F-2000 IC cation-measurement software (Hitachi, San Jose, CA). The excitation and emission values used were as follows (in nm): Quin-2: Ex 340, Em 490; CTC: Ex 390, Em 530; calcium green: Ex 488, Em 530; Indo-1: Ex 350, Em1 405, Em2 480; Fura-2: Ex1 340, Ex2 380, Em 510. Samples of 770 μL were loaded into 1.0-mL methacrylate UV/vis cuvettes (Fisher Scientific, Pittsburgh, PA) and stirred using a small magnetic stir bar and built-in stir plate. Excitation and emission maxima were obtained using the wavelength scan function of the F-2000 fluorometer, scanning at 1 nm/s.

Chilling samples

Two circulating water baths were connected by insulated tubing to the jacketed cuvette holder and separated from each other by valves. The heated water bath was held at 45°C, and the chilled water bath was held at −5°C. Sample temperature was monitored with an Omega (Stamford, CT) microprocessor thermometer using a thin K-type thermocouple wire threaded into the cuvette, but not disrupting the beam. To cool samples, the heated bath valve was closed and the chilled bath valve was opened. Reversal of this process warmed the samples.

pH and viscosity

A Corning pH/ion analyzer 255 was used to measure pH of the solutions and samples. Viscosity was monitored by measuring the flow rate of a given solution from a 100-mL reservoir in a buret through a borosilicate capillary tube (75 mm × 1 mm (I.D.)).

Fluorescence lifetimes

Multifrequency phase-modulation fluorimetry was used to measure the excited-state lifetimes of all five dyes under both calcium-free and calcium-saturated conditions at 5 and 40°C. All time-resolved fluorescence intensity decay kinetics were measured in the frequency domain using an SLM-AMINCO 48000 multiharmonic Fourier (MHF) spectrofluorometer (Spectronic Instruments). The instrument and its capabilities have been described in detail elsewhere (Wang et al., 1995). For these particular experiments, we used a cw argon-ion laser (Coherent, Innova 90−6) operating at 488.0 nm as the excitation source for calcium green and a 325.0 nm He-Cd laser (Omnichrome Series 74XA) for all other probes. In both cases, an appropriate interference filter (Oriel, Stratford, CT) was placed in the excitation beam path to prevent extraneous plasma-tube superradiance or Rayleigh scatter from reaching the detection system. The sample fluorescence was monitored in the typical L-format after passing through either a 515-nm (argon-ion) or a 345-nm (He-Cd) longpass filter. The Pockels cell modulator was operated at a 5 MHz base repetition rate. Typically, data sets were acquired for 60 s between 5 and 150 MHz (30 total frequencies). At least 10 discrete multifrequency data sets were acquired for each sample under a given set of experimental conditions. For the excited-state intensity decay measurements, we used a dilute solution of rhodamine 6G dissolved in water or Me_2POPOP in ethanol as the reference lifetime standards with assigned lifetime values of 3.85 ns (Heitz and Bright, 1995) and 1.45 ns (Lakowicz, 1983), respectively. Magic angle polarization conditions were used for all excited-state intensity decay measurements to eliminate bias arising from fluorophore rotational reorientation.

In the frequency domain, the experimental measurables for excited-state fluorescence intensity decay experiments are the frequency-dependent phase angle (Θ) and demodulation factor (M). The excited-state fluorescence lifetimes are recovered from the frequency-domain data by using a commercially available nonlinear least squares software package (Globals Unlimited, Urbana, IL) (Beechem et al., 1991). In all data analyses, we

used the true uncertainty in each datum as the frequency-dependent weighting factor. Background and supplemental details on the theory of phase-modulation fluorescence can be found elsewhere (Bright, 1995; Bright et al., 1990). The time-resolved fluorescence intensity decay $I(t)$ can generally be sufficiently described by a stretched exponential of the form (Bright, 1995; Bright et al., 1990),

$$I(t) = \sum_{i=1}^{n} f_i e^{-t/\tau_i}, \quad (1)$$

in which f_i is the fractional intensity of the ith emissive species possessing excited-state lifetime τ_i. For a multiexponential decay (e.g., $n = 2$), the arithmetic mean excited-state lifetime ($\langle\tau\rangle$) can be calculated as

$$\langle\tau\rangle = \sum_{i=1}^{n} f_i \tau_i. \quad (2)$$

RESULTS AND DISCUSSION

Effect of temperature on fluorescence

The reported calcium concentration is roughly proportional to fluorescence intensity for the single-wavelength dyes, and roughly proportional to the ratio of fluorescence intensity at two wavelengths for the ratiometric dyes. If fluorescence is affected by temperature, it follows that variations in temperature would affect the calcium concentration value reported by the dye. In fact, fluorescence intensities of the single-wavelength dyes (CTC, Quin-2, and calcium green) increased by as much as twofold due to a shift from 40 to 5°C (Fig. 1). This result cannot be ascribed to the effect of temperature on K_d, because it is seen under both fully saturated (1 mM Ca^{2+}) and calcium-free (10 mM EGTA) conditions. The extent of the fluorescence gain due to the temperature shift varied widely among the three dyes tested, with CTC and Quin-2 showing much more dramatic increases than calcium green. Calcium green, therefore, may be a more ideal choice for an investigation in which temperature is being changed than either CTC or Quin-2.

Although the effect of temperature on fluorescence intensity of the single-wavelength dyes is dramatic, the resulting effect on calcium-sensing is likely to be smaller than one might predict on the basis of Fig. 1. Because both the bound and free forms of the dye are affected by temperature, and because calcium determination is based on a self-ratioing equation (Tsien and Pozzan, 1989), part of the temperature effect will be controlled by this calculation. However, the effects of temperature on the bound and free forms are not identical, and a 20–30% difference remains between the F_{min}/F_{max} ratios at 5 versus 40°C. Thus, the effect of temperature on fluorescence intensity is one potential source of error in investigations involving variable temperatures. The other main source of error is, of course, the effect of temperature on K_d of the dye. As stated above, this effect has already been thoroughly investigated, and thus is not addressed in the current study. Nevertheless, both must be considered to obtain reliable data.

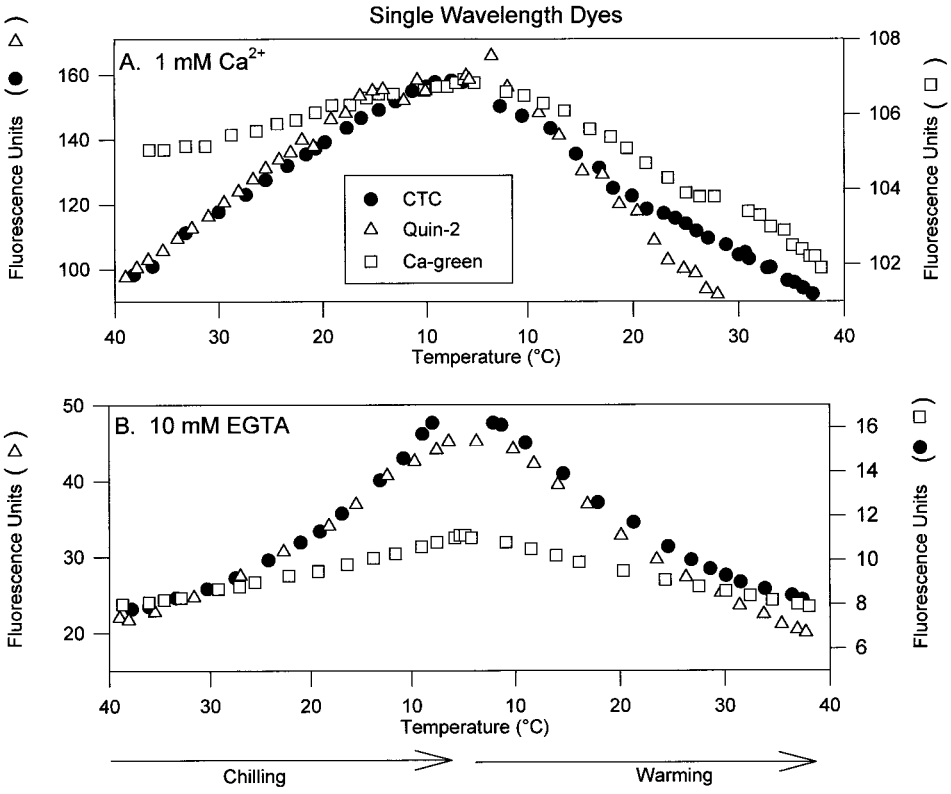

FIGURE 1 Dependence of fluorescence on temperature for single-wavelength calcium-sensitive dyes measured in the presence of (A) 1 mM $CaCl_2$, and (*B*) 10 mM EGTA for Quin-2 (△), chlortetracycline (●), and calcium green dextran (□). Calcium green, for which a second *y* axis is shown at the right, has a much smaller increase in fluorescence intensity than either Quin-2 or chlortetracycline.

Fluorescence intensities of the ratiometric dyes were also affected by temperature. Indo-1 and Fura-2 were examined in both calcium-saturated and calcium-free conditions by chilling and rewarming the samples. Indo-1 showed a decrease in the fluorescence ratio (Em 405/480) at low temperature under both conditions, although the effect was more pronounced in the calcium-free samples (Fig. 2 *A*). Nevertheless, a decrease of 10–25% in the fluorescence ratio could have a significant effect on the reported calcium concentration.

In most cases, Fura-2 showed a decrease in the fluorescence ratio (Ex 340/380) at low temperature as well (Fig. 2 *B*). A complicating factor, however, was that, under calcium-saturating conditions, the magnitude—and even direction—of the change in fluorescence ratio depended on the age and spectroscopic history of the sample (which was not the case for Indo-1). This phenomenon is illustrated in the inset to Fig. 2 *B*. A Fura-2 sample (1 mM Ca^{2+}) was repeatedly temperature-scanned over a period of 10 days. On the initial scan, the fluorescence ratio increased by 7% during the shift from 40 to 5°C. On all subsequent scans, however, the fluorescence ratio decreased at low temperature, and the magnitude of this effect became more pronounced with each successive scan. This finding emphasizes the importance of careful controls during variable temperature investigations. The degree to which the fluorescence ratio varies with temperature depends on the type of dye, the dye concentration, calcium concentration, and, in some cases, age of sample.

These many and complex variables might help to explain the apparent discrepancy of these results with one study that reported no change in the fluorescence ratio during a temperature shift (Rink et al., 1982a). Such effects have been seen previously, however. For instance, Lakowicz notes that Fura-2 may undergo phototransformation producing noncalcium-sensitive subpopulations (Lakowicz et al., 1994; Becker and Fay, 1987). Clearly, the photobleaching has been documented to some extent, but this effect has not been widely understood in the community.

As is the case for the single wavelength dyes, then, temperature has a considerable effect on the fluorescence of the ratiometric dyes as well, as evidenced by the temperature-induced trends in the λ1/λ2 ratios. These effects must be considered, along with the effects of temperature on dye K_d, to measure free calcium accurately.

Possible mechanisms

To illuminate the causes of the effects of temperature on the calcium-sensitive dyes, we investigated several possible

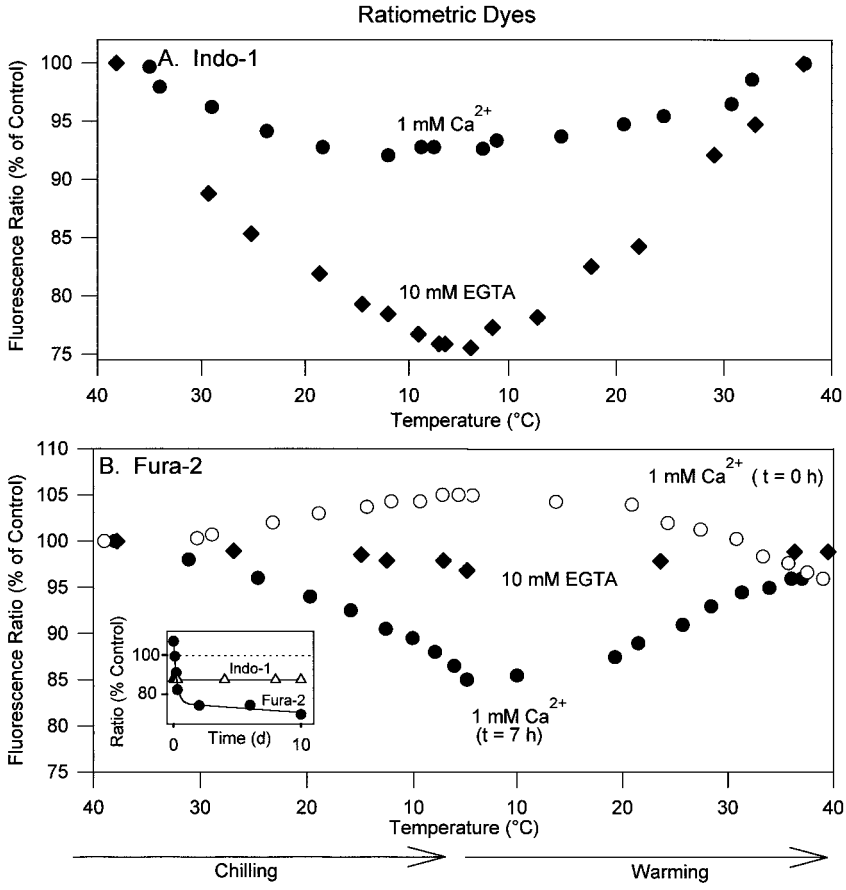

FIGURE 2 (*A*) Dependence on temperature of the λ1/λ2 ratio for Indo-1 in the presence of 1 mM $CaCl_2$ (●), or 10 mM EGTA (♦). Ratios are reported as percentages of the 40°C values. (*B*) Dependence on temperature of the λ1/λ2 ratio for Fura-2 in the presence of 1 mM $CaCl_2$ or 10 mM EGTA (♦). Calcium-saturated Fura-2 scans shown include the initial scan (t_0) (○) and the 4th scan (t = 7 h) (●). Ratios are reported as percentages of the 40°C values. Dependence of the temperature effect on sample age is shown in the inset for both Fura-2 (●) and Indo-1 (△). A dashed line is shown at the 100% (no change) level for reference.

mechanisms using two different methods. One method involved mimicking the physical changes that occur in the samples during cooling while holding temperature constant. The alternate method was to check for specific changes in fluorescence characteristics that could be responsible for the effects of low temperature, and determine if they did indeed occur when temperature was reduced.

pH

The well-known phenomenon of increasing pH at decreasing temperature is responsible for important changes regarding the binding equilibria of fluorescent calcium chelators (Lattanzio, 1990; Lattanzio and Bartschat, 1991; Shuttleworth and Thompson, 1991). To determine if the pH changes caused by cooling were responsible for any of the fluorescence changes as well, the pH change was mimicked in constant temperature samples. During chilling, the pH of PBS increased from 7.0 (at 37°C) to 7.3 (at 5°C). When the pH of samples, held constant at 37°C, was shifted from pH 7.0 to 7.3 by the addition of base, there was no significant change in fluorescence intensity for most of the five dyes tested (Table 1). Of the single-wavelength dyes, only CTC showed an increase in fluorescence intensity with an increase in pH. However, the shift to pH 7.3 was not sufficient to cause the full fluorescence rise seen during the shift to 5°C (~60% increase). Nevertheless, the elevation in pH could contribute to the fluorescence intensity changes seen in CTC with temperature. This trend is not an absolute, however, and each system must be measured separately. Evidence for this comes from measurements of CTC at higher concentrations. For instance, CTC when measured at 380 μM, showed the opposite trend (i.e., a decrease in fluorescence intensity as pH increased), possibly due to the existence of aggregated species.

The dual wavelength dyes showed virtually no change in the fluorescence ratios between pH 7.0 and 7.3 (see Table 1). However, larger pH changes (i.e., to 8.3 and above) did cause the ratios of both Fura-2 and Indo-1 to decline. Thus, although under the conditions of the current investigation, pH was not primarily responsible for the effects seen, it is still an important consideration due to its effects on both fluorescence and binding equilibria. For intracellular measurements, however, this concern becomes less important, because living cells regulate their intracellular pH closely and rapidly.

TABLE 1 Fluorescence intensity changes resulting from a shift from pH 7.0 to 7.3 at constant temperature in the presence of 1 mM Ca^{2+}

Dye	pH 7.0	pH 7.3	% Change
CTC*	10.3 ± 0.3	12.7 ± 0.2	+22
Quin-2*	44.9 ± 1.8	44.8 ± 1.2	−0.2
Calcium green*	25.0 ± 3.5	19.6 ± 3.7	−22
Fura-2†	31.0 ± 0.3	31.1 ± 0.3	−0.3
Indo-1†	2.81 ± 0.03	2.84 ± 0.1	+1.1

*Fluorescence intensity reported (in AFU).
†λ1/λ2 fluorescence ratio reported.

Viscosity

Another important physical parameter of solutions that changes with temperature is viscosity. Because viscosity has been shown to be correlated inversely with oxygen quenching (Eftink and Ghiron, 1987) and positively correlated with quantum yield (Eastman and Rosa, 1968), we tested the calcium-sensitive dyes to determine if the increase in viscosity at low temperature were responsible for the temperature-based effects on fluorescence. The higher viscosity of the low-temperature samples was mimicked by the addition of sucrose to the buffer. We determined that a solution of PBS with 1.1 M sucrose had approximately the same viscosity at 37°C as the PBS alone had at 5°C (1.5 cp [CRC, 1984]) as described in Materials and Methods. The effects of viscosity on fluorescence intensity are shown in Fig. 3. In the case of the single-wavelength dyes (Fig. 3 *A*), fluorescence intensities in the high viscosity PBS were not significantly higher than in the PBS. In fact, in the case of CTC, the intensity in the high viscosity PBS was significantly lower than in the PBS alone. Clearly, viscosity is not responsible for the elevated fluorescence intensity at hypothermic temperatures for the single wavelength dyes.

Viscosity is also not responsible for the effects of temperature on the fluorescence of the ratiometric dyes. As shown in Fig. 3 *B*, although fluorescence intensity increases can be seen for Indo-1, the changes are not significant for Fura-2. However, for these viscosity effects on Indo-1 to cause a decrease in the λ1/λ2 fluorescence ratio (as is seen in Fig. 2), the fluorescence intensity increase due to viscosity would have to be greater for wavelength-2 than for wavelength-1. In fact, the opposite is true. Thus, viscosity cannot be responsible in the case of Indo-1. It has been noted (Poenie, 1990; Busa, 1992) that high viscosity can reduce the λ1/λ2 ratio for Fura-2, but the viscosity at which these effects were seen was very large (15 cp) compared to this study (1.5 cp), and, as stated, under the current conditions, the effect of viscosity on Fura-2 was not significant. However, aside from the effects on fluorescence mentioned above, changes in viscosity can also affect the binding kinetics of the dyes (Kao and Tsien, 1988). Thus, viscosity is similar to pH in that it can have important consequences in regard to both binding characteristics and fluorescence values.

Quenching

Fluorescence quenching due to molecular oxygen is directly proportional to temperature (Eftink and Ghiron, 1987). Therefore, it is possible that the fluorescence increases seen

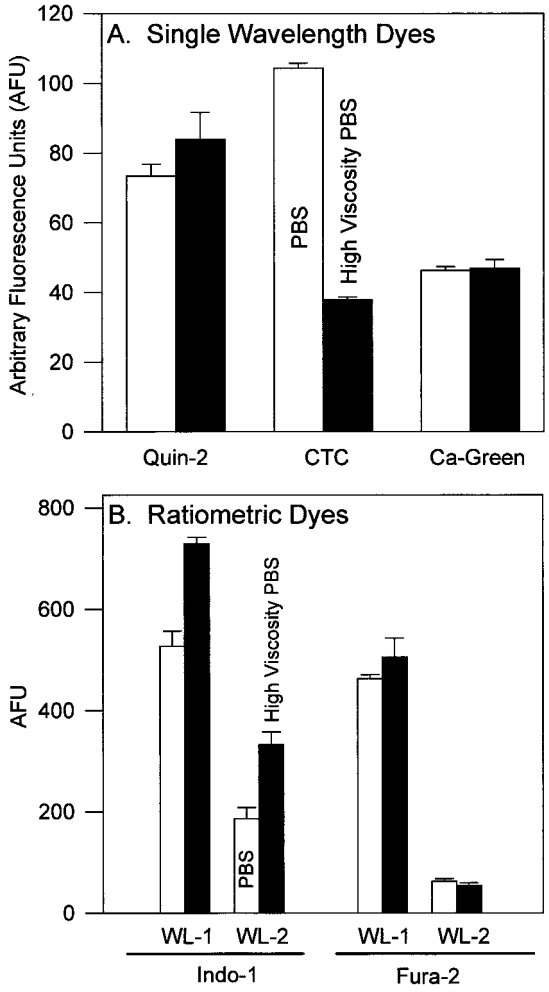

FIGURE 3 Dependence of fluorescence intensity on viscosity for (*A*) single-wavelength and (*B*) ratiometric calcium-sensitive dyes measured in the presence of 1 mM $CaCl_2$. The high viscosity PBS buffer contained 1.1 M sucrose, which raised the viscosity at 37°C to that of PBS buffer alone at 5°C.

at low temperature might be due to a drop in oxygen quenching. Samples of all five dyes in the presence or absence of calcium (1 mM $CaCl_2$ or 10 mM EGTA) were tested in sealed cuvettes, either with or without a 20-min purge (by bubbling with N_2 gas) to eliminate dissolved oxygen (Saltiel et al., 1993). There were no significant differences between the temperature effects of purged and nonpurged samples for any of the dyes tested (Table 2). This is expected due to the short excited-state lifetimes of the probes involved. Thus, modulation of oxygen quenching cannot be responsible for the effects of temperature seen on the calcium-sensitive dyes.

Spectral shifts

Changes in excitation or emission maxima with temperature have been noted for several fluorophores (Demchenko and Ladokhin, 1988; Law, 1994; Suelter, 1967). To investigate the possibility that such shifts could be responsible for the effects of temperature on the fluorescent dyes, we ran wavelength scans in the presence and absence of calcium at 40 and 5°C. Most of the maxima were not shifted by temperature (Table 3). However, a 6-nm red shift did appear for the emission maximum of calcium-saturated Fura-2 (493 to 499 nm) when the temperature was reduced from 40 to 5°C, as has been previously described (Paltauf-Doburzynska and Graier, 1997). In addition, a 4-nm red shift (469 to 473 nm) was evident for the emission maximum of Indo-1 under calcium-free conditions resulting from the same temperature reduction.

In the case of Fura-2, the shift to 499 nm brings the +Ca ($\lambda 1$) emission maximum closer to the measured emission wavelength (510 nm). This could theoretically have the effect of raising the fluorescence intensity at the +Ca value, which would tend to increase the ratio I_{+Ca}/I_{-Ca} at low temperature. Mimicking the temperature-induced spectral shift at constant temperature by bringing the value of the measured emission wavelength 6 nm closer to the +Ca emission maximum (from 510 to 504 nm) caused a minimal change in the fluorescence ratio (approx. +1% change, positive as predicted).

In the case of Indo-1, in contrast, the shift in emission maximum from 469 to 473 nm under calcium-free conditions brings the −Ca maximum closer to the −Ca measured value of 480 nm. This would tend to increase the fluorescence intensity at the −Ca value, which would tend to decrease the I_{+Ca}/I_{-Ca} ratio at low temperature. This is, in fact, what occurs when Indo-1 is chilled (see Fig. 2). Mimicking the spectral shift at constant temperature by reducing the measured −Ca emission value from 480 to 476 nm, which brings it 4 nm closer to the −Ca emission maximum, did cause a significant (~10%) decrease in the fluorescence ratio. One reason for the larger change seen for Indo-1 than for Fura-2 in this regard is that, for Indo-1, the fluorescence maximum, which is shifted by temperature, is the same one that is shifted by calcium binding (emission). In contrast, for Fura-2, the emission maximum is shifted by temperature, whereas the excitation maximum is shifted by calcium binding. This could lead to a less pronounced effect of spectral shifts for Fura-2. Nevertheless, this mechanism may contribute to the change in the fluorescence ratio seen at low temperature for both ratiometric dyes.

Fluorescence lifetimes

There is a considerable body of literature showing that excited-state lifetimes of many fluorescent molecules are lengthened as temperature decreases (Drain et al., 1998; Kumke et al., 1997; Young et al., 1997; Bark and Force, 1993; van den Zegel et al., 1984) in correlation with a decrease in the rates of nonradiative decay (e.g., decreased coupling to vibronic bath modes) (Lam et al., 1998; Young

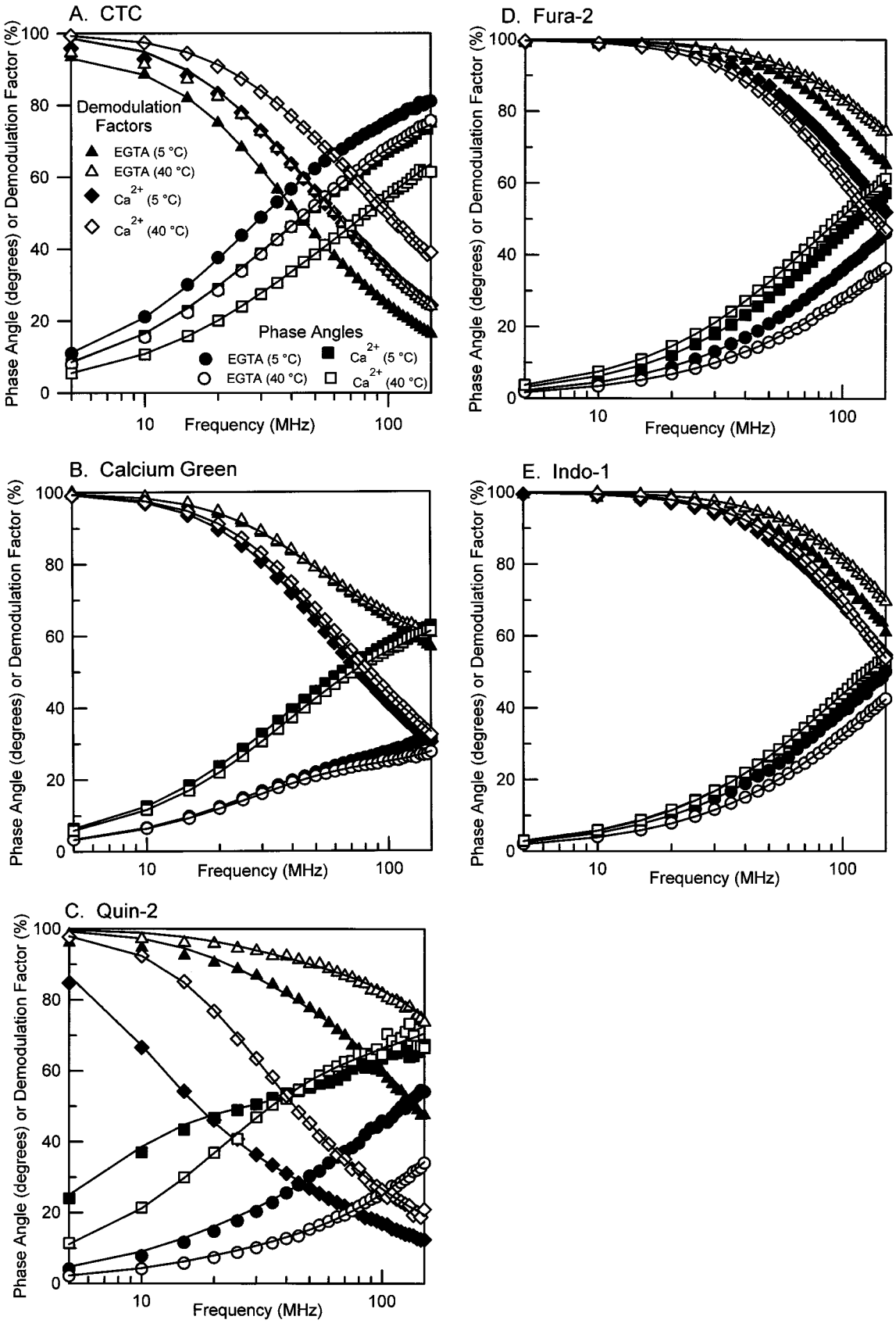
A. CTC
Demodulation Factors
EGTA (5 °C)
EGTA (40 °C)
Ca2+ (5 °C)
Ca2+ (40 °C)
Phase Angles
EGTA (5 °C)
EGTA (40 °C)
Ca2+ (5 °C)
Ca2+ (40 °C)
Phase Angle (degrees) or Demodulation Factor (%)
Frequency (MHz)
B. Calcium Green
C. Quin-2
D. Fura-2
E. Indo-1

TABLE 2 Changes in fluorescence caused by reducing the temperature of the sample from 40 to 5°C in either oxygen-rich or oxygen-purged conditions in the absence of Ca^{2+}

Dye	Oxygen-rich (%)	Oxygen-purged (%)
CTC*	+245	+244
Quin-2*	+224	+223
Calcium green*	+143	+144
Fura-2†	−5.4	−5.4
Indo-1†	−24	−24

*Effect on fluorescence intensity reported.
†Effect on λ1/λ2 fluorescence ratio reported.

TABLE 3 Fluorescence maxima taken in the presence and absence of calcium at 40 and 5°C

	40°C		5°C	
Probe	Ex	Em	Ex	Em
1 mM Ca^{2+}				
CTC	374 (± 1)	522 (± 1)	375 (± 2)	524 (± 2)
Quin-2	331 (± 0)	495 (± 1)	331 (± 1)	494 (± 1)
Ca-green	504 (± 0)	530 (± 1)	504 (± 0)	528 (± 0)
Fura-2	337 (± 1)	*493 (± 1)	337 (± 1)	*499 (± 1)
Indo-1	329 (± 1)	400 (± 1)	330 (± 1)	400 (± 1)
10 mM EGTA				
CTC	342 (± 1)	425 (± 1)	342 (± 1)	424 (± 1)
Quin-2	357 (± 1)	488 (± 1)	357 (± 1)	487 (± 1)
Ca-green	504 (± 0)	529 (± 0)	504 (± 0)	528 (± 0)
Fura-2	364 (± 0)	507 (± 1)	366 (± 1)	507 (± 0)
Indo-1	346 (± 1)	*469 (± 1)	347 (± 1)	*473 (± 1)

Values are means (±SD) for 3–5 replicates for each type of dye.
*Significant differences between 40 and 5°C.

et al., 1997; Giri, 1992). This leads to a net increase in fluorescence intensity (i.e., quantum yield), because, when decay back to the ground state does occur, it is more likely through emission of a photon. To determine if this trend held for the calcium-sensitive dyes as well, the lifetimes of all five dyes were measured using multifrequency phase-modulation fluorimetry, under both calcium-free and calcium-saturated conditions, at 40 and 5°C (Fig. 4). In all cases, the data were best described by a double exponential decay, as shown in Table 4. Multiple exponential decays have been reported before for Quin-2 and Indo-1 (Lakowicz et al., 1992, 1994; Szmacinski et al., 1993), and excited-state reactions were suggested as a possible cause (Szmacinski et al., 1993).

Mean excited-state decay times were then calculated (Eq. 2), based on the fractional contribution of the decay arising from each discrete lifetime, and are summarized in Table 5. As expected, in nearly every case, the excited-state lifetime, at a given Ca^{2+} condition, was longer at the lower temperature, the only exception occurring for calcium-saturated Fura-2 for which no change was observed with a decrease in temperature.

In the case of the single-wavelength dyes, this result is readily interpreted. At low temperatures, the excited-state lifetimes are longer, generally leading to increased fluorescence intensity. The least pronounced change occurs in calcium green, which also shows the smallest intensity increase at 5°C (see Fig. 1). Similarly, the most pronounced effect of temperature on fluorescence lifetime is seen in Quin-2, which also shows one of the most dramatic effects of temperature on intensity for any of the dyes tested. This is a significant result because the excited-state lifetime of Quin-2 can itself be used to monitor intracellular calcium concentration (Lakowicz et al., 1994; Miyoshi et al., 1991) stemming from its sensitivity to calcium binding. Clearly, without detailed prior knowledge of its temperature-dependent behavior, this probe would be a poor choice for applications involving variable temperature.

The ratiometric dyes show a similar trend, in that reduced temperature leads to increases in the fluorescence lifetimes. However, the effects are much more pronounced in the calcium-free condition than in the calcium-saturated condition. The increase in fluorescence intensity at the calcium-free values would cause a decrease in the I_{+Ca}/I_{-Ca} ratio at low temperature, which correlates well with experimental results for Indo-1. In the case of Fura-2, the effect of temperature predicted by the exited-state lifetimes (decrease in fluorescence ratio) contrasts with that predicted by the spectral shifts (increase in fluorescence ratio). The balance between these two aspects of the system may determine the overall effect of temperature on the fluorescence ratio of Fura-2. The effect of temperature on the excited-state lifetimes of the calcium-sensitive dyes is, therefore, likely to be important in determining the overall effect of temperature on fluorescence.

Can fluorescent dyes be used to measure Ca^{2+} at variable temperatures?

Although the calcium-sensitive dyes show a strong dependence of fluorescence on temperature, they can still be used to make reasonably accurate measurements of calcium concentration at variable temperatures, as long as the investigation controls for both the intensity and K_d effects. The

FIGURE 4 Multifrequency phase and modulation data for (*A*) chlorotetracyline, (*B*) calcium green, (*C*) Quin-2, (*D*) Fura-2, and (*E*) Indo-1. Phase angles are shown for calcium-free samples at 5°C (●) and 40°C (○), and also for calcium-saturated samples at 5°C (■) and 40°C (□). Demodulation factors are shown for calcium-free samples at 5°C (▲) and 40°C (△) and also for calcium-saturated samples at 5°C (◆) and 40°C (◇). The solid lines denote the best double-exponential fits to the experimental data. These data were analyzed as described in Materials and Methods to determine the excited-state lifetimes for each fluorophore (see Tables 4 and 5).

TABLE 4 Excited-state decay parameters for selected calcium-sensitive fluorophores at 5 and 40°C

		1 mM Ca^{2+}				10 mM EGTA			
Dye	T (°C)	f_1	τ_1 (ns)	τ_2 (ns)	X^2	f_1	τ_1 (ns)	τ_2 (ns)	X^2
CTC	5	0.89	4.67	1.32	1.00	0.46	8.64	5.06	1.27
	40	0.49	4.52	1.72	0.32	0.45	6.75	3.28	0.74
Quin-2	5	0.92	16.27	2.09	0.61	0.25	6.20	1.45	1.61
	40	0.91	6.73	1.07	0.22	0.13	5.24	0.69	0.48
Calcium Green	5	0.87	4.01	0.73	0.45	0.47	3.58	0.40	0.56
	40	0.86	3.70	0.70	0.86	0.46	3.60	0.33	0.16
Fura-2	5	0.94	1.81	0.44	0.68	0.68	1.54	0.64	0.53
	40	0.94	1.82	0.27	1.30	0.34	1.68	0.60	0.62
Indo-1	5	0.79	1.91	0.63	0.82	0.12	3.00	1.20	0.18
	40	0.95	1.68	0.09	0.10	0.55	1.42	0.69	0.24

Lifetimes shown are the best fit for the experimental data, f_1 is the fractional intensity of τ_1, and X^2 is the reduced chi square.

most appropriate method for accomplishing this task involves conducting all data manipulation at the sample temperature of interest. For instance, Indo-1 and Fura-2 can both be used to accurately measure calcium at several different temperatures, as long as the maximal calcium reading (1 mM Ca^{2+}) and the minimal calcium reading (10 mM EGTA) are taken at the same temperature as the measured calcium reading, and that the correct temperature-adjusted K_d is used for the calculation.

A second method that can be used to measure the calcium concentration in samples at variable temperature is better suited for experiments that require continuous measurement rather than measurements at discrete time and temperature points. In such cases, it is important to find a dye that is nontemperature-dependent in the range of interest. For example, in the physiological range, the calcium concentration reported by Indo-1 shows little temperature sensitivity between 20 and 5°C, although it is highly temperature-dependent in the range from 20 to 40°C (Oliver et al., 1999). Therefore, it is possible to estimate continuous changes in calcium concentration as a function of temperature between 20 and 5°C using Indo-1 (Oliver et al., 1999), but would not be recommended in other more temperature-dependent ranges. Even under favorable circumstances of low sensitivity of fluorescence to temperature, however, it is still critical to control for the effects of temperature on K_d, and determine that this type of artifact is not responsible for any measured effect on intracellular calcium.

TABLE 5 Excited-state lifetimes for five probes, under calcium-saturated and calcium-free conditions, at 5 and 40°C

Dye	T (°C)	$\langle\tau\rangle$ (ns) (1 mM Ca^{2+})	$\langle\tau\rangle$ (ns) (10 mM EGTA)
CTC	5	4.30	6.71
	40	3.09	4.84
Quin-2	5	15.14	2.64
	40	6.22	1.28
Ca Green	5	3.58	1.89
	40	3.28	1.83
Fura-2	5	1.73	1.25
	40	1.73	0.97
Indo-1	5	1.64	1.42
	40	1.60	1.09

All showed a biexponential decay, and are reported as the arithmetic mean excited-state lifetime $\langle\tau\rangle$, calculated as described in Materials and Methods.

In summary, the fluorescence behavior for all five calcium-sensitive probes studied revealed some degree of temperature sensitivity. Increase in excited-state lifetimes along with reduced rates of nonradiative decay, are likely to contribute strongly to such effects. In addition, increasing pH may play a role in the effect of temperature on CTC, and spectral shifts probably also contribute to the effect of temperature on the ratiometric probes. Nevertheless, the intermolecular interactions in a fluorescent solution are extremely complex, and the effects of decreasing fluorescence intensity at elevated temperatures are likely to be the result of many different factors, including some not considered in the current study. Although the general effect of temperature on fluorescence intensity is well known to photophysics specialists, the use of the calcium-sensitive dyes is becoming extremely common for researchers specializing in many other disciplines as well. Therefore, we have attempted to describe some of the photophysical effects of temperature on these dyes in an effort to draw attention to the importance of considering this issue if temperature is used as an independent variable in experiments using the calcium-sensitive dyes.

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