

Caffeine Interaction with Fluorescent Calcium Indicator Dyes

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ABSTRACT We report that caffeine, in millimolar concentrations, interacts strongly with four common calcium indicator dyes: mag-fura-2, magnesium green, fura-2, and fluo-3. Fluorescence intensities are either noticeably enhanced (mag-fura-2, fura-2) or diminished (magnesium green, fluo-3). The caffeine-induced changes in the fluorescence spectra are clearly distinct from those of metal ion binding at the indicator chelation sites. Binding affinities for calcium of either mag-fura-2 or magnesium green increased only slightly in the presence of caffeine. Caffeine also alters the fluorescence intensities of two other fluorescent dyes lacking a chelation site, fluorescein and sulforhodamine 101, implicating the fluorophore itself as the interaction site for caffeine. In the absence of caffeine, variation of solution hydrophobicity by means of water/dioxane mixtures yielded results similar to those for caffeine. These observations suggest that hydrophobic substances, in general, can alter dye fluorescence in a dye-specific manner. For the particular case of caffeine, and perhaps other commonly used pharmacological agents, the dye interactions can seriously distort fluorescence measurements of intracellular ion concentrations with metal indicator dyes.

INTRODUCTION

Calcium indicator dyes offer several advantages over alternative means of assessing intracellular calcium activity, such as calcium electrodes. Indicator dyes are comparatively unintrusive, they discriminate effectively between Ca^{2+} and Mg^{2+} , they come in a range of affinities, and they permit spatially and temporally resolved measurements of calcium distributions within cells, particularly when used in conjunction with confocal detection and/or two-photon excitation. Measurements with ratiometric dyes (Grynkiewicz et al., 1985) reduce problems resulting from dye bleaching and path length variation. Finally, the advent of acetoxymethyl ester (AM) forms (Tsien, 1981) of many of these dyes made it possible to load indicators into isolated cells or tissues without prior membrane permeabilization. This multitude of advantages has made calcium indicator dyes the method of choice for the study of intracellular calcium kinetics (Nuccitelli, 1994). Few applications have seen as much traffic as the investigation of calcium stores, particularly those responding to methylxanthines (e.g., Weber, 1968; Pessah et al., 1987; Thayer et al., 1988). Caffeine-sensitive calcium stores (microsomes, endoplasmic reticulum, calciosomes), for example, have been described in a variety of neuronal cell bodies, including dorsal root ganglion cells (Neering and McBurney, 1984; Usachev et al., 1993; Shmigol et al., 1995), rat sympathetic neurons (Thayer et al., 1988), cultured rat cerebellar Purkinje cells (Brorson et al., 1991), rat cerebellar Purkinje neurons in brain slices (Kano et al., 1995), as well as in a variety of

other cell types (e.g., Volpe et al., 1988). Caffeine-induced mobilization of intracellular calcium has been assessed, most often by the use of fluorescent tetracarboxylate Ca-indicator dyes (e.g., Thayer et al., 1988).

There are, however, a variety of problems and corrections associated with quantitative fluorescence measurements of intracellular calcium. Among these are phototoxicity, which tends to preclude long-term measurements, and the likelihood of significant changes in the calcium affinities of the dyes, as observed, e.g., in the intracellular environment of muscle fibers (Konishi et al., 1988).

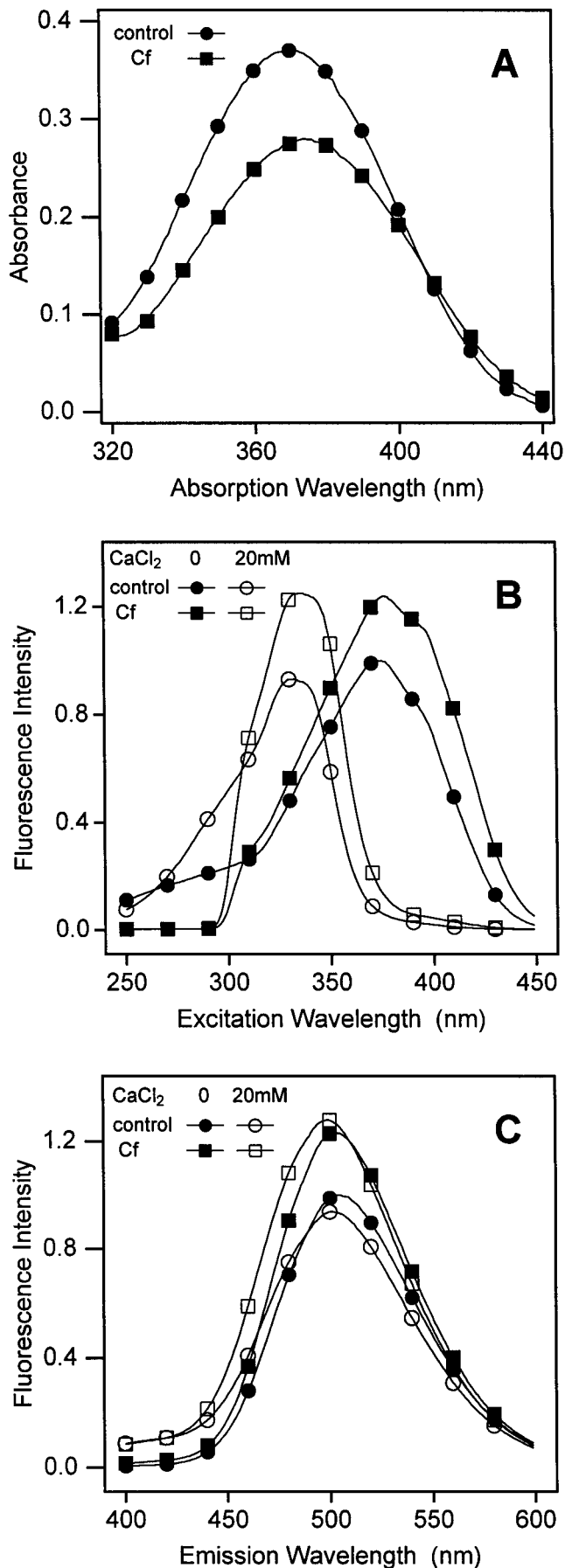
Here we report a potential pitfall in the use of fluorescent indicator dyes to assess physiological effects of caffeine. We have been studying the relation of Ca^{2+} changes to an intrinsic optical signal (Salzberg et al., 1985) detectable during exocytosis from nerve terminals of the mammalian neurohypophysis (Muschol and Salzberg, 1998). Both of these signals are noticeably altered in the presence of millimolar concentrations of caffeine. Incongruities in the apparent physiological response to caffeine reported by two different calcium indicators, mag-fura-2 and magnesium green, suggested that we examine the direct effects of caffeine on the fluorescence of the calcium indicator dyes. Other investigators have reported that caffeine modifies the absorbance or fluorescence of individual calcium indicators (Best and Abramcheck, 1985; O'Neill et al., 1990; O'Neill and Eisner, 1990; Donoso et al., 1994; Islam et al., 1995). In the present paper we provide a detailed qualitative and quantitative description of the effects of caffeine on several important calcium indicator dyes. We have also attempted to locate the interaction site of caffeine and to elucidate the type of interaction underlying the observed effects on indicator fluorescence. We find that caffeine interacts primarily with dyes' fluorophores via nonspecific, hydrophobic interactions. Thus effects of caffeine on dye fluorescence—rather than isolated aberrations—are undoubtedly the norm.

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More generally, our data suggest that dye fluorescence will be modulated by a wide range of hydrophobic substances, including many drugs.

MATERIALS AND METHODS

Stock solutions of mag-fura-2 (tetrapotassium salt), magnesium green (pentapotassium salt), fluo-3 (pentaammonium salt), and fura-2 (tetrapotassium salt) (Molecular Probes, Eugene, OR) were typically prepared at 1 mM concentration in distilled water. The dye stock was added to measurement solutions (see below) in a dilution of 1:100.

The response of all four dyes to caffeine was determined as a function of caffeine concentrations between 0 and 60 mM. Two stock solutions were prepared. Stock A contained 10 μ M dye, 10 μ M or 5 mM EGTA, 130 mM KCl, 20 mM HEPES, adjusted to pH 7.4 with NaOH. Stock B, in addition, contained 60 mM caffeine. Individual samples were obtained via mixtures of the two stocks. For mag-fura-2, fura-2, and magnesium green, the titrations were performed with the dye in the Ca^{2+} -free state. For fluo-3 the Ca^{2+} -saturated state was selected for its higher fluorescence intensity. For the latter experiments, 20 mM CaCl_2 was substituted for EGTA in both stock solutions.

The effects of caffeine on calcium affinities were investigated with mag-fura-2 and magnesium green. Dye affinities for Ca^{2+} were first determined in the absence of caffeine. Stock A contained 10 μ M dye, 15 mM citric acid, or ATP (two low-affinity Ca^{2+} buffers), 130 mM KCl, 20 mM HEPES, adjusted to pH 7.4 with NaOH. Citric acid or ATP was chosen because their relatively low binding affinities permit reliable buffering of calcium over the range of concentrations used in our experiments. Addition of 10–20 mM CaCl_2 to stock A, with readjustment of pH, yielded stock B. A series of solutions with various concentrations of total calcium, $[\text{Ca}_t^{2+}]$, was obtained from titrations of the two stocks. Based on $[\text{Ca}_t^{2+}]$, the citric acid (or ATP) concentration, and a calcium/citric acid (calcium/ATP) dissociation constant K_d of 458 μ M (160 μ M), the resulting values for $[\text{Ca}^{2+}]$ were calculated using the public domain program winmaxc v1.7 (Bers et al., 1994). K_d values of all calcium buffers (citric acid, ATP, HEDTA, EGTA) were taken from Martel and Smith (1974) and extrapolated to our experimental conditions ($T = 20^\circ\text{C}$, pH 7.4, ionic strength = 160) within winmaxc. Measurements were repeated after 30 mM caffeine was added to both stock solutions before titration with the desired $[\text{Ca}_t^{2+}]$. With the exception of Ca^{2+} indicator dyes (Molecular Probes) and the two different grades of caffeine (Sigma Chemicals, St. Louis, MO, and Aldrich Chemical Comp., Milwaukee, WI), all chemicals were purchased from Fluka Chemical Corp. (Ronkonkoma, NY) and were microselect grade.

Absorption spectra were measured with a lambda-40 UV/vis spectrometer (Perkin-Elmer, Norwalk, CT). All fluorescence spectra were collected with a PTI spectrofluorometer (Photon Technology International, Monmouth Junction, NJ), with the spectral bandwidth of the excitation monochromator (DeltaRAM) adjusted to 5 nm. The solution temperature was maintained at 20°C with a water circulator. The wavelength calibration of the unit was confirmed with a dysprosium-activated yttrium aluminum garnet (DYAG) crystal, which can be used as a fluorescence wavelength standard (PTI). However, there is no absolute calibration of the wavelength-dependent output intensity. The fluorescence spectra are, therefore, a convolution of dye response and instrument characteristics. For this reason, all fluorescence spectra were normalized to the peak fluorescence

FIGURE 1 Absorption and fluorescence spectra of 10 μ M mag-fura-2 in 0 mM or 20 mM CaCl_2 , 10 μ M EGTA, 130 mM KCl, 20 mM HEPES (pH 7.4), with either 0 mM or 30 mM caffeine. (A) Absorption spectra in 0 mM CaCl_2 only. (B) Fluorescence excitation spectra with fluorescence emission monitored at $\lambda_{em} = 505$ nm. (C) Fluorescence emission spectra of mag-fura-2, excited at either $\lambda_{ex} = 374$ nm (0 mM CaCl_2) or $\lambda_{ex} = 332$ nm (20 mM CaCl_2). Fluorescence intensities are normalized to $F_{0,max}$, the maximum intensity of the (excitation or emission) spectrum in the 0 mM CaCl_2 control trace. $T = 20^\circ\text{C}$.

intensity of the corresponding calcium-free and caffeine-free (excitation or emission) spectrum.

Caffeine and calcium binding data were both analyzed using a 1:1 binding model for two-state dyes (for details, see Grynkiewicz et al., 1985). For total caffeine concentrations $[Cf_t]$ well in excess of the total dye concentration $[D_t]$, the fractional fluorescence change induced by caffeine at any given wavelength can be expressed as

$$\frac{\Delta F}{F_0} = \frac{\Delta \epsilon_\lambda}{\epsilon_{f,\lambda}[D_t]} \frac{[Cf_t]}{[Cf_t] + K_d^{Cf}}. \quad (1)$$

Here $\Delta F = F - F_0$, $F_0 = \epsilon_{f,\lambda}[D_t]$ is the fluorescence intensity in the absence of caffeine, and K_d^{Cf} is the apparent caffeine dissociation constant. The coefficients $\epsilon_{b,\lambda}$, $\epsilon_{f,\lambda}$ describe the fluorescence responses of the caffeine-bound and caffeine-free states, respectively, and $\Delta \epsilon_\lambda = (\epsilon_{b,\lambda} - \epsilon_{f,\lambda})$. The corresponding relation used for analyzing the calcium binding data is

$$F_\lambda = [D_t] \left(\epsilon_{f,\lambda} + \Delta \epsilon_\lambda \frac{[Ca]}{[Ca] + K_d^{Ca}} \right). \quad (2)$$

The free calcium concentration $[Ca]$, as determined from total calcium and total calcium buffer concentration, is the independent variable. The term $\epsilon_{b,\lambda}$ in $\Delta \epsilon_\lambda = (\epsilon_{b,\lambda} - \epsilon_{f,\lambda})$ now refers to the calcium-bound state DCa, instead. Using a nonlinear fitting procedure adapted from Konishi et al. (1991), we exploited the whole spectrum to extract values for K_d^{Ca} , the calcium-dye dissociation constant. The suitability of these models for describing caffeine-dye and caffeine-calcium-dye interactions will be critically examined below.

RESULTS

Caffeine titration spectra

Mag-fura-2

Fig. 1 documents the changes induced by 30 mM caffeine on the absorption, as well as fluorescence excitation and emission spectra of mag-fura-2, also known as furaptra (Raju et al., 1989). Absorption measurements of mag-fura-2 reveal that 30 mM caffeine reduces the intrinsic dye absorption by 25%, while shifting the absorption peak from 369 to 374 nm. In contrast, the excitation spectra (Fig. 1 B) of the Ca^{2+} -bound and the Ca^{2+} -free states both show strong fluorescence enhancements of 30% and 23% at their respective excitation peaks of 332 nm and 374 nm. The peak positions of both excitation spectra become slightly redshifted by ~ 4 nm. Overall, the long-wavelength side of the excitation spectrum extends as much as 15 nm further into the red, and a weak shoulder at 400 nm in the Ca^{2+} -free spectrum is noticeably enhanced in the presence of caffeine. These spectral changes are also associated with a shift from 347 to 352 nm in the isosbestic wavelength λ_{iso} (the wavelength at which the fluorescence intensity of the Ca^{2+} -bound state equals that of the Ca^{2+} -free dye state). Note that the sharp drop of the excitation spectra at wavelengths below 310 nm in the presence of caffeine does not correspond to a direct effect of caffeine on the dye spectrum. It originates, instead, from caffeine's intrinsic UV absorption (data not shown), which diminishes dye excitation. The fluorescence emission spectra of mag-fura-2 (Fig. 1 C) exhibit enhancements of their amplitudes similar to those observed in the excitation spectra, with no discernible shifts

in the relative position of the emission peaks. The overall effect of caffeine on mag-fura-2 is therefore twofold: a decrease in dye absorption of $\sim 25\%$, compensated by an even more pronounced increase in quantum yield. Given the drop in peak absorption with the concurrent increase in peak emission ($\geq 23\%$), 30 mM caffeine enhances the quantum yield of mag-fura-2 by at least 67%.

Fig. 2 A shows a caffeine titration of fluorescence excitation spectra obtained with the Ca^{2+} -free form of mag-fura-2. The difference spectra of Fig. 2 B highlight the caffeine-induced spectral changes in the data. Note that ΔF

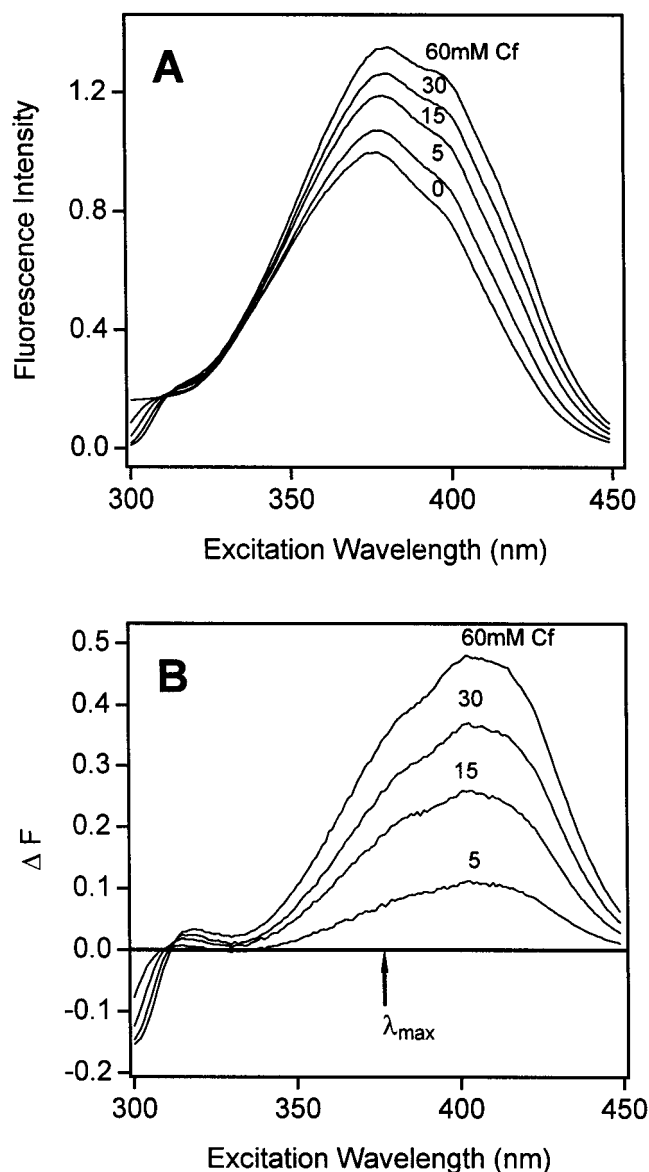


FIGURE 2 (A) Fluorescence excitation spectra of $10 \mu M$ mag-fura-2 in 0 mM $CaCl_2$, $10 \mu M$ EGTA, 130 mM KCl, 20 mM HEPES (pH 7.4), and 0, 5, 15, 30, or 60 mM caffeine (Cf). Fluorescence emission was collected at $\lambda_{em} = 505$ nm. (B) Difference spectra obtained from A after subtraction of excitation spectrum with 0 mM Cf. Fluorescence intensities are normalized to $F_{0,max}$, the maximum intensity of the Ca^{2+} -free control spectrum. $T = 20^\circ C$.

peaks around 400 nm, and not at the peak position of F ($\lambda = 374$ nm), emphasizing the preferential enhancement of the long-wavelength shoulder of the excitation spectrum by caffeine. In Fig. 3 the fractional change in the 374-nm fluorescence of the excitation spectrum is plotted against the caffeine concentration. The solid line represents a fit through this change, based on a 1:1 binding reaction of caffeine and the dye. The fit is good, with an apparent caffeine dissociation constant (K_d^{Cf}) of 28 mM. Given the nonspecific nature of the caffeine-dye interaction (see below), the conspicuously good agreement between this simple model and the data is probably fortuitous and should not be interpreted as compelling evidence for the existence of a caffeine-dye complex of fixed stoichiometry.

Fura-2

The effects of increasing concentrations of caffeine on the excitation spectrum of the Ca^{2+} -free state of fura-2 are shown in Fig. 4. There is a redshift in the excitation peak of 4 nm from 367 to 371 nm. The largest relative change in the difference spectrum (see Fig. 4 B) occurs ~ 15 nm to the red of the excitation peak. Analysis of the fractional change in peak fluorescence (not shown) yields an apparent value for K_d^{Cf} of 21 mM. There is a clear resemblance between the responses of fura-2 and mag-fura-2 to caffeine, despite their different ion chelation structures (tetracarboxylate for fura-2, tricarboxylate for mag-fura-2; see Haugland, 1996) and different dissociation constants for calcium (see Table 1).

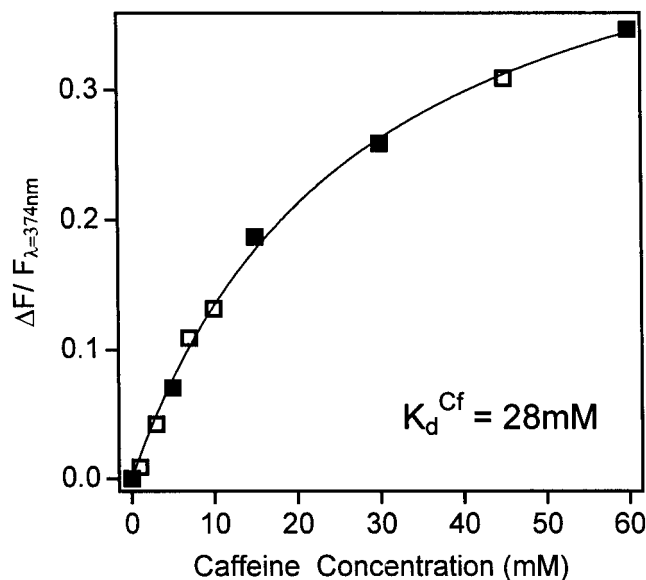


FIGURE 3 Fractional change in fluorescence, $\Delta F/F$, for mag-fura-2 (10 μM) in 10 μM EGTA, 130 mM KCl, 20 mM HEPES (pH 7.4), and 0, 1, 3, 5, 7, 10, 15, 30, 45, or 60 mM caffeine. $\Delta F/F$ was determined at the wavelength of the excitation peak, $\lambda_{\text{max}} = 374$ nm. The solid squares indicate the data selected for display in Fig. 2. The continuous line represents a fit through the data with a 1:1 binding model (see text for details), yielding a best-fit value of $K_d^{Cf} = 28$ mM. $T = 20^\circ\text{C}$.

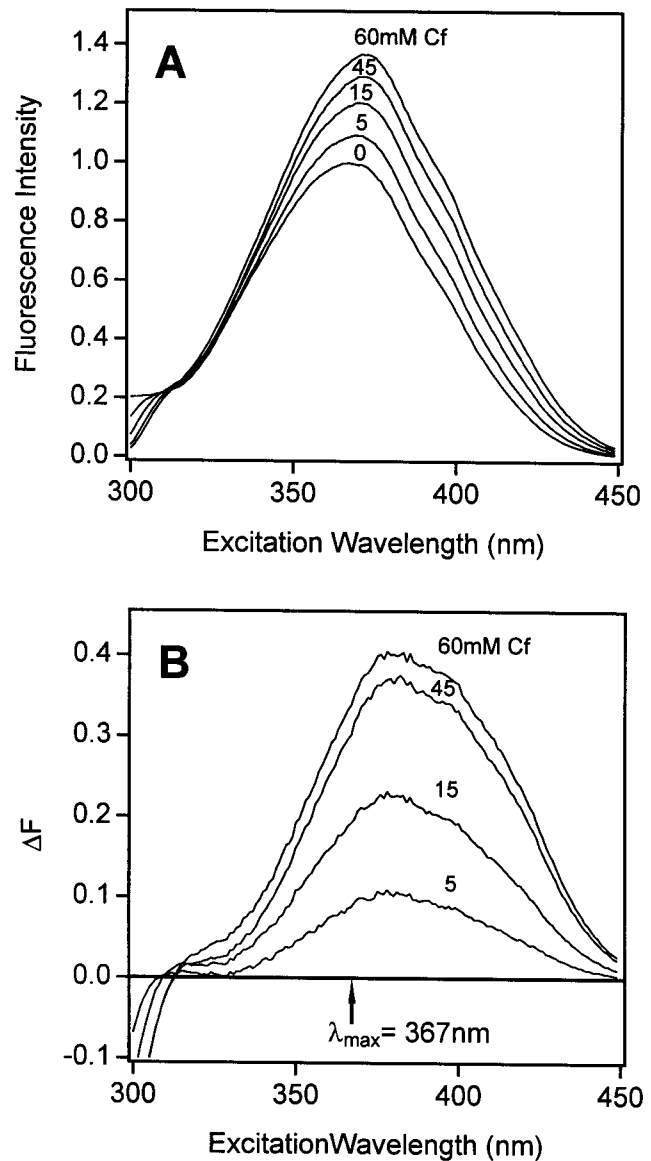


FIGURE 4 (A) Fluorescence excitation spectra of fura-2 (20 μM) in 5 mM EGTA, 130 mM KCl, 20 mM HEPES (pH 7.4), and caffeine concentrations of 0, 5, 15, 45, or 60 mM. Emission was monitored at $\lambda = 540$ nm. Fluorescence intensities are normalized to $F_{0,\text{max}}$, the maximum intensity of the Ca^{2+} -free spectrum. (B) Difference spectra obtained from A after subtraction of 0 mM Cf - spectrum. $T = 20^\circ\text{C}$.

Magnesium green

Absorption and fluorescence spectra for magnesium green in the Ca^{2+} -free state and at various caffeine concentrations are illustrated in Fig. 5. Caffeine decreases the peak absorption of magnesium green by $\sim 15\%$ and redshifts the peak position from 507 to 512 nm (Fig. 5 A). At the same time, caffeine decreases the fluorescence excitation intensity of magnesium green (Fig. 5 B), as well as the emission intensity (Fig. 5 C). (Binding of divalent ions, in contrast, increases the fluorescence intensity of this indicator dye.) In the excitation spectrum (Fig. 5 B), the intensity of the secondary peak at 475 nm relative to that of the main peak

TABLE 1 Summary of dye properties and measurement results

Indicator	Mag-fura-2	Fura-2	Magn. green	Fluo-3	Indo-1
Mol. wt.	587	832	915.9	855	840
λ_{em} (nm)	505	505	532	530	485/410 [#]
λ_{ex} (nm)	374/332*	362/335 [#]	508	497	349/331 [#]
K_d^{Ca} (μ M)	$50 \pm 4^*$	$0.224,^{#} 0.145^{\S}$	$16 \pm 2^*$	0.390^{\S}	$0.250,^{#} 0.230^{\S}$
K_d^{Ca} (μ M) (with caffeine)	$43 \pm 4^*$	—	$13 \pm 2^*$	—	—
K_d^{Cr} (mM)	28^*	21^*	8^*	7^*	$3.9/18^{\parallel}$

*Values determined in this work.

[#]Gryniewicz et al. (1985).

^{\S}Haugland (1996).

^{\parallel}O'Neill et al. (1990). First value *in vitro*, second *in vivo*.

at 510 nm is noticeably reduced by caffeine. At the highest caffeine concentration, the peak position of the excitation spectrum is only marginally redshifted (by 2 nm). The corresponding emission spectrum (Fig. 5 C) is redshifted by as much as 9 nm, which results in a readily visible change in the color of the dye. Combining the decrease in absorption (15%) and fluorescence (38%), the reduction in quantum efficiency for magnesium green in 30 mM caffeine equals $(1 - 0.62/0.85)$ or 27%. Analysis of the fractional fluorescence change as a function of caffeine concentration (not shown) yields an apparent value of K_d^{Cr} of 8 mM.

Fluo-3

With fluo-3, the caffeine titration data were collected in the presence of 20 mM $CaCl_2$, i.e., in the Ca^{2+} -saturated state. Fig. 6 A shows that the fluorescence excitation intensity decreases with increasing caffeine concentration. The position of the excitation peak remains fairly constant, but there is a significant suppression of the fluorescence peak at 475 nm compared to those at longer wavelengths. The emission spectra exhibit a steady decline in fluorescence intensity, coupled with a pronounced redshift (14 nm at 60 mM caffeine) in the peak position (Fig. 6 B). The spectral response of fluo-3 to caffeine closely resembles that of magnesium green. The apparent value of the K_d^{Cr} of fluo-3 is 7 mM.

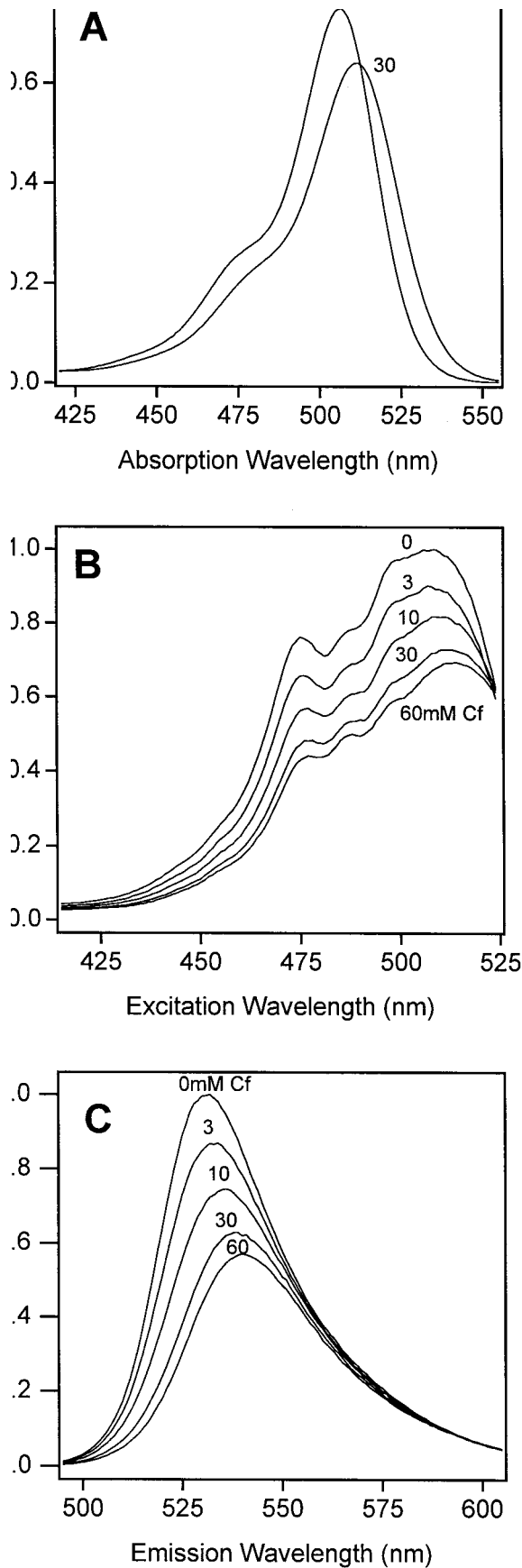
Is caffeine the culprit?

Several tests were performed to establish caffeine-dye interactions as the origin of the observed changes in dye fluorescence. Caffeine from two different suppliers (Sigma Chemicals, St. Louis, MO, and Aldrich Chemicals, Milwaukee, WI) and two different purity levels (anhydrous versus food grade) yielded the same effect (data not shown). Contamination of caffeine by divalent metal ions (e.g., Mg^{2+} , Ba^{2+} , Sr^{2+}) that would compete with Ca^{2+} at the dyes' chelation sites should result in spectral responses comparable to those with Ca^{2+} (Raju et al., 1989; Murray and Kotlikoff, 1989; Xu-Friedman and Regehr, 1999). The spectral response to caffeine exhibited by all dyes is clearly distinct from the spectral response to divalents. We also

tested whether caffeine altered dye fluorescence indirectly through an effect on a third solution component. Systematic substitutions of the calcium buffers (HEDTA, EGTA, or ATP instead of citric acid) or the pH buffer (piperazine-*N,N'*-bis(2-ethanesulfonic acid instead of HEPES) or omission of KCl did not affect the observed caffeine-dye interaction. Shifts in pH resulting from caffeine addition were less than 0.05 pH units at the maximum concentration of 60 mM and were corrected before any measurements. Hence our experiments strongly support the interpretation that the fluorescence changes result from a direct interaction of caffeine with the indicator dyes.

Alterations of Ca^{2+} binding by caffeine

The effect of 30 mM caffeine on the dissociation constant of Ca^{2+} was determined for two of the dyes, mag-fura-2 and magnesium green. Fluorescence excitation spectra of mag-fura-2 at a series of free calcium concentrations [Ca^{2+}] were obtained in the absence or presence of 30 mM caffeine. Calcium dissociation constants, K_d^{Ca} , were derived by fitting families of spectra, with and without caffeine, to a standard 1:1 Ca^{2+} :dye stoichiometry (Gryniewicz et al., 1985). Applying this model implicitly presumes that competitive binding of caffeine and Ca^{2+} to the same binding site can be excluded. In the subsequent discussion we will provide arguments that support this assumption. Representative results from these nonlinear fits to the spectra of mag-fura-2 and magnesium green are shown in Figs. 7 and 8, respectively. Ca^{2+} affinities obtained with this procedure for mag-fura-2 and magnesium green, with or without 30 mM caffeine, are listed in Table 1. In the absence of caffeine, the dissociation constant of mag-fura-2, $K_d^{Ca} = 50 \pm 4 \mu$ M, agrees well with published results of 44 μ M (Konishi et al., 1991). In contrast, the best-fit binding affinity of magnesium green, $16 \pm 2 \mu$ M, is somewhat higher than the value of 7 μ M reported by Zhao et al. (1996) (see also Xu-Friedman and Regehr, 1999). Note that the affinity is close to typical levels of Ca^{2+} contamination. Therefore, the choice of a suitable chelator to control calcium levels is important. Because our solution conditions differ in both temperature (20°C versus 16°C) and pH (7.4 versus 7.0) from those of Zhao et al., the observed discrepancies could



also arise from a strong dependence of K_d^{Ca} on either parameter. Other potential sources of errors are the values of free calcium, derived numerically from $[\text{Ca}_t^{2+}]$, total calcium buffer concentration, and, most important, accurate values for K_d , the calcium-buffer dissociation constants. The latter depend critically on extrapolations of tabulated calcium buffer affinities to our experimental conditions.

Caffeine-dye interaction site

Several lines of evidence indicate that caffeine interacts with all of the dyes in this study at a site distinct from the Ca^{2+} chelation site. First, caffeine alters the dye fluorescence spectra of all dyes in this study in a manner clearly distinct from that of metal ion binding. This is most obvious for the ratiometric dyes fura-2 and mag-fura-2. The addition of caffeine enhances the amplitudes of their excitation spectra and redshifts their peaks independently of the state of metal ion binding. In contrast, the main effect of ion binding on the excitation spectra of the dye is a blueshift (e.g., Fig. 7 A). Notice also that the apparent binding affinity of mag-fura-2 for caffeine is 28 mM, whereas the affinity for Ca^{2+} is 50 μM . Therefore, when the two of them are present at comparable concentrations, Ca^{2+} would easily displace caffeine from a common binding site on the dye, rendering it ineffective. This is clearly not consistent with the data in Fig. 1 B, where caffeine shows an even bigger effect on the amplitude of the dye fluorescence in the Ca^{2+} -bound state than in the Ca^{2+} -free state. Similar arguments apply to fluo-3 and magnesium green (caffeine reduces their fluorescence intensity, ion binding increases it). In addition, the pronounced redshift of their emission peaks with caffeine (Figs. 6 B and 5 C) is not observed upon ion binding (data not shown). The modest increase in Ca^{2+} affinity of both mag-fura-2 and magnesium green in the presence of caffeine is therefore likely to be the result of secondary effects on the Ca^{2+} -binding site.

It is worthwhile to compare our observations to previous reports on caffeine interactions with calcium indicator dyes. Best and Abramcheck (1985) showed that the metallochromic indicator dye arsenazo-III (Brown et al., 1975) decreases its absorption upon the addition of caffeine. In contrast, ion binding by arsenazo-III either increases or decreases dye absorption, depending on the observation wavelength. The authors nevertheless interpreted their data

FIGURE 5 Absorption and fluorescence spectra of magnesium green (10 μM) in 5 mM EGTA, 130 mM KCl, 20 mM HEPES (pH 7.4). (A) Absorption spectra in either 0 or 30 mM caffeine. (B) Fluorescence excitation spectrum of magnesium green at caffeine concentrations of 0, 3, 10, 30, or 60 mM. Emission was monitored at the peak of the corresponding emission spectrum (Note: The long-wavelength portions of the excitation spectra are clipped because of the small Stokes shift between the excitation and emission peaks.) (C) Fluorescence emission spectra of samples in B with dye excitation at $\lambda_{\text{em}} = 475$ nm. All fluorescence intensities are normalized to $F_{0,\text{max}}$, the maximum intensity of the Ca^{2+} -free (excitation or emission) spectrum. $T = 20^\circ\text{C}$.

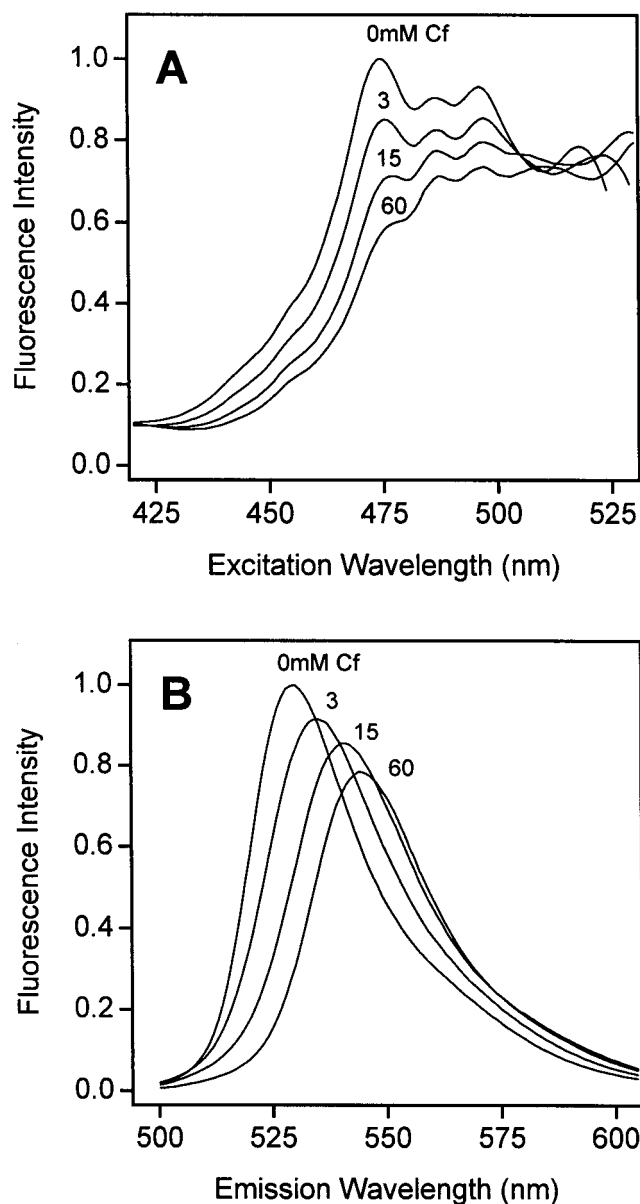


FIGURE 6 (A) Fluorescence excitation spectra of fluo-3 (20 μM) in 10 mM CaCl₂, 130 mM KCl, 20 mM HEPES (pH 7.4), and caffeine concentrations of 0, 3, 15, or 60 mM. Fluorescence emission was monitored at the peak of the corresponding emission spectrum, λ_{em} = 530–544 nm. (B) Fluorescence emission spectra corresponding to the samples in A, with dye excitation at λ_{ex} = 497 nm. Fluorescence intensities are normalized to $F_{0,max}$, the maximum intensity of the Ca²⁺-free spectrum. $T = 20^{\circ}\text{C}$.

in terms of a 1:1 binding of caffeine at the ion chelation site. No competitive binding data were provided. Based on the different spectral responses to caffeine versus metal ions, the absorption dye arsenazo-III may well have a separate site for caffeine interaction, as also seen with the fluorescent indicators in this study. Similarly, caffeine induced quenching of indo-1 fluorescence, independent of emission wavelength and calcium concentration (O'Neill et al., 1990; O'Neill and Eisner, 1990; Donoso et al., 1994). Again, this response is clearly different from the emission wavelength shift observed upon binding of calcium to indo-1. Finally,

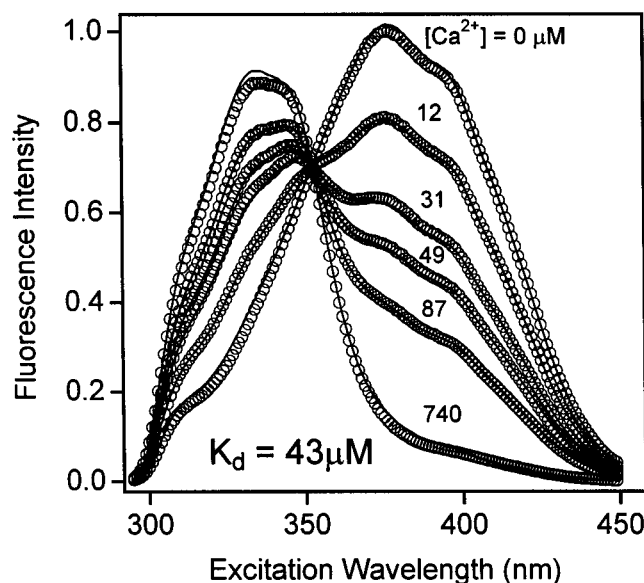


FIGURE 7 Fluorescence excitation spectra of mag-fura-2 (10 μM) in 30 mM caffeine, 15 mM citric acid, 130 mM KCl, 20 mM HEPES (pH 7.4), and concentrations of free calcium [Ca²⁺] of 0, 12, 31, 49, 87, and 740 μM. Fluorescence emission was monitored at λ_{em} = 505 nm. The solid curves are fits through these spectra with a 1:1 binding model (see text for details). The dye/calcium dissociation constant derived from this fit is $K_d = 43 \mu\text{M}$.

the distinct enhancement of fura-2 fluorescence by caffeine, detailed here, was previously noted by Islam et al. (1995) but missed by O'Neill et al. (1990).

The obvious similarities in the caffeine response of mag-fura-2 to that of fura-2 on one hand, and of magnesium-green to fluo-3 on the other hand, correlate well with the structural similarities of their respective fluorophores (Haugland, 1996) and hint at the fluorophore as the site for the caffeine-dye interaction. We tested this hypothesis with fluorescein, which is closely related to the fluorophore of fluo-3 and magnesium green. The spectral response of fluorescein to 30 mM caffeine is shown in Fig. 9. Again, the main effect of caffeine is to reduce fluorescein's fluorescence intensity. In the excitation spectra (Fig. 9 A), the intensity ratio of the 475-nm shoulder to the main peak is decreased, and there is a marginal redshift. The emission spectra (Fig. 9 B) reproduce the noticeable redshift of the peak position observed with fluo-3 and magnesium green. We are not aware of a commercially available fluorophore, without a chelation site, that is comparable in structure to fura-2 and mag-fura-2. However, we examined another fluorescent dye, sulforhodamine 101, with results similar to those with fluorescein (data not shown). All of these observations clearly implicate the dyes' fluorophore as the interaction site of caffeine.

Origin of the caffeine-dye interaction

Effects on dye fluorescence from drug-dye interactions are not confined to caffeine. As suggested by Best and Abram-

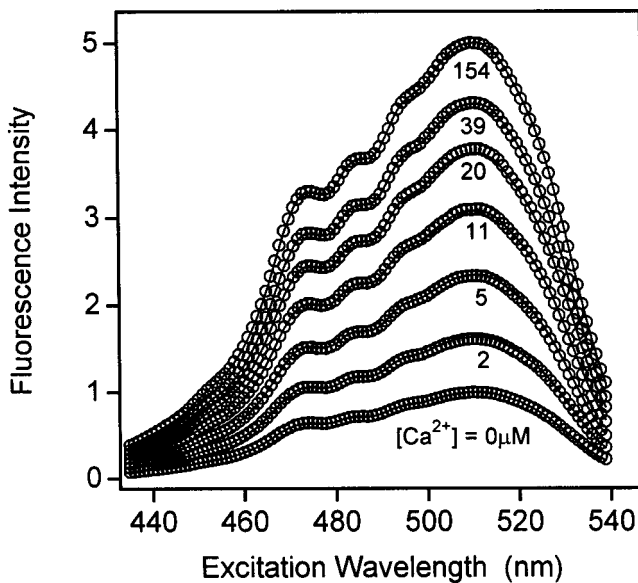


FIGURE 8 Fluorescence excitation spectra of magnesium green (10 μM) in 30 mM caffeine, 15 mM ATP, 130 mM KCl, 20 mM HEPES (pH 7.4), and concentrations of free calcium $[\text{Ca}^{2+}]$ of 0, 2, 5, 11, 20, 39, and 154 μM . Fluorescence emission was monitored at $\lambda_{\text{em}} = 545 \text{ nm}$. The solid curves are fits through these spectra with a 1:1 binding model (see text for details). The dye/calcium dissociation constant derived from this fit is $K_d = 12 \mu\text{M}$.

check (1985), and later observed by Donoso et al. (1994), other methylxanthines yield similar effects. This is confirmed here by the response of fluorescein to 2 mM theobromine (curve labeled *Tb* in Fig. 9). Given the diverse chemical structures of the dyes responding to methylxanthines, a possible mechanism for the observed effects on dye fluorescence is a nonspecific, hydrophobic interaction (Tanford, 1973). Hence we attempted to replicate the caffeine-dye interaction by using a hydrophobic mixture of 80% water/20% dioxane. Fig. 9 also compares the responses of fluorescein in the two solutions. Superposition of the excitation spectra of fluorescein in 20% dioxane (dashed line in Fig. 9) and 30 mM caffeine emphasizes the similarity in the spectral response. Fig. 10 compares the corresponding response of mag-fura-2 to caffeine and dioxane. Again, dioxane induces an intensity enhancement similar to that produced by caffeine. Thus an increase in solution hydrophobicity indeed appears to mimic the observed caffeine-dye effects. The 1000-fold higher molar concentration of dioxane required to reproduce an effect equivalent to caffeine's indicates how effectively caffeine alters the hydrophobicity in the microenvironment of the dye. This may result from either binding or enhanced local concentrations of caffeine within the dye's immediate vicinity.

DISCUSSION

Implications for optical calcium measurements

The direct interaction of caffeine with calcium indicator dyes occurs within the same range of millimolar concentra-

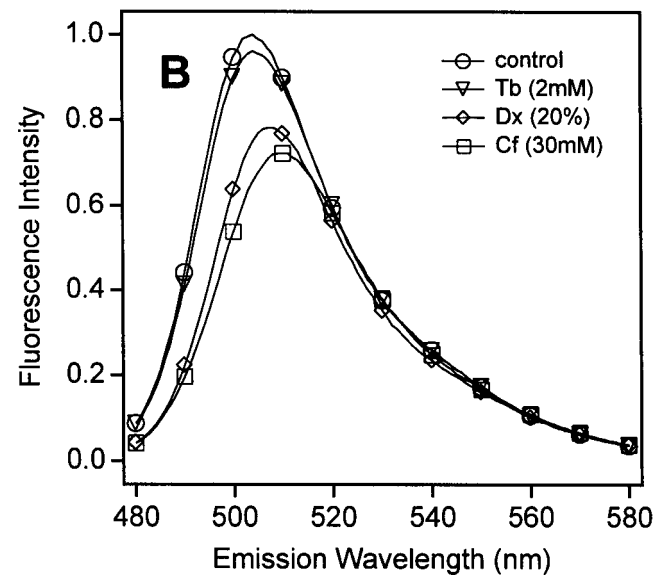
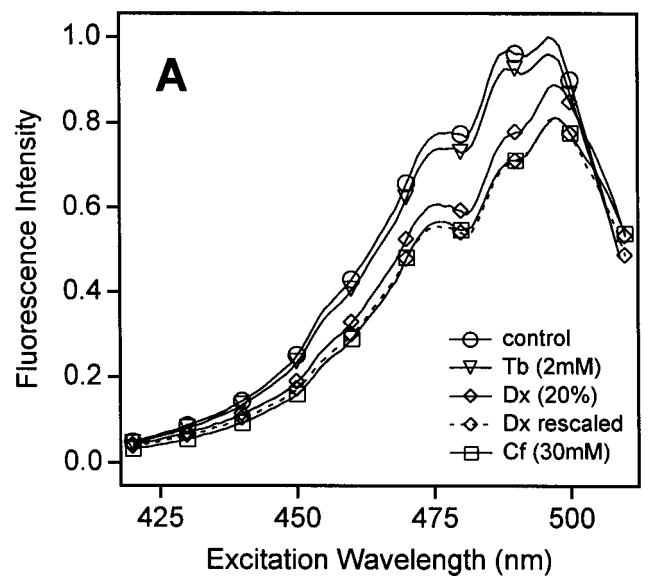


FIGURE 9 Fluorescence spectra of fluorescein (10 μM) in 130 mM KCl, 20 mM HEPES (pH 7.4), and subsequent addition of either 30 mM caffeine (Cf) or 2 mM theobromine (Tb), or substitution of 20 vol% of aqueous medium by dioxane (Dx). (A) Fluorescence excitation spectra with emission monitored at the peak of the corresponding emission spectrum $\lambda_{\text{em}} = 513\text{--}520 \text{ nm}$. (B) Fluorescence emission spectra corresponding to the samples in B, with $\lambda_{\text{ex}} = 488 \text{ nm}$.

tions used to elicit physiological responses (see references mentioned in Introduction). Therefore, these interactions have important, dye-specific implications for observations of caffeine-mediated physiological changes in cell calcium levels.

It is important to note that the apparent caffeine affinities vary significantly between different fluorophores. For example, mag-fura-2 and fura-2 have lower apparent caffeine affinities than the fluorescein derivatives magnesium green and fluo-3. Thus a higher apparent K_d^{Cf} might be regarded as a simple criterion for limiting the effects of caffeine on

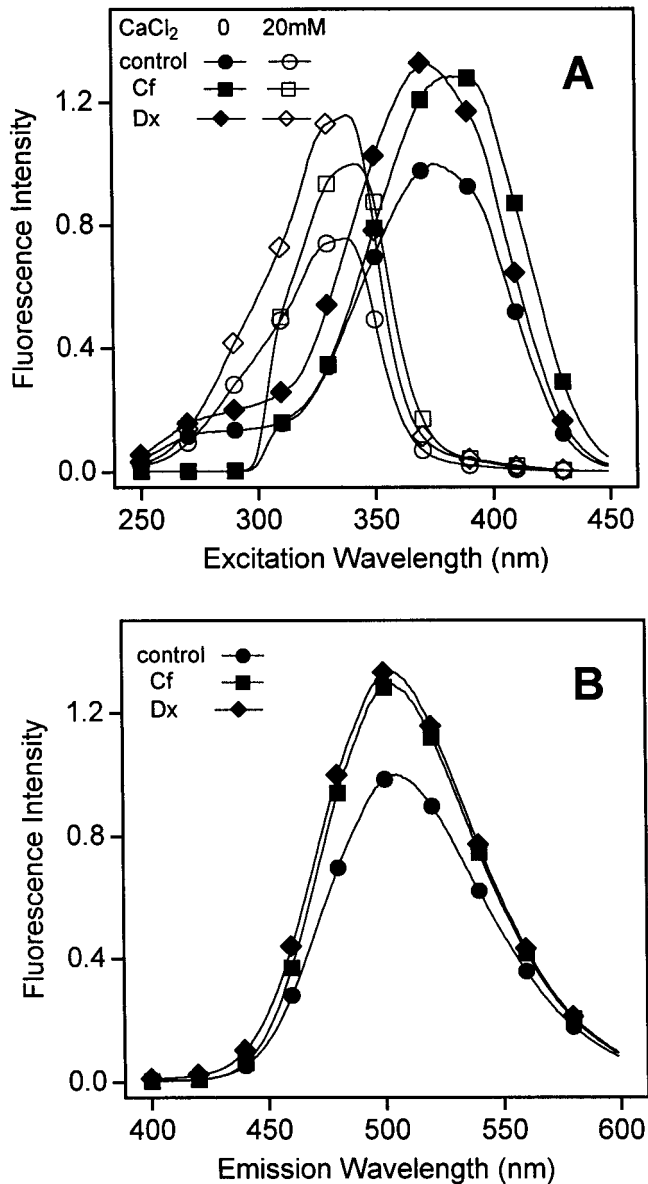


FIGURE 10 Fluorescence spectra of 10 μM mag-fura-2 in 130 mM KCl, 10 μM EGTA, 20 mM HEPES (pH 7.4) in 0 mM CaCl₂ (Ca²⁺-free state) or 20 mM CaCl₂ (Ca²⁺-saturated state), with the addition of 30 mM caffeine (Cf) or the substitution of 20 vol% of aqueous medium by dioxane (Dx). (A) Fluorescence excitation spectra with emission monitored at the peak of the corresponding emission spectrum λ_{em} = 505 nm. (B) Fluorescence emission spectra of the calcium-free samples in A, with λ_{ex} = 374 nm (for clarity, the 20 mM CaCl₂ spectra were omitted).

fluorescent indicator measurements of ion concentration. However, several other factors determine the extent to which caffeine interferes with measurements of [Ca²⁺]_i in a specific experiment. Equally important is the fact that the quantity F_{\max}/F_{\min} (the ratio of the maximum to minimum indicator fluorescence in zero and saturating [Ca²⁺]_i) is altered in the presence of caffeine. The situation is further complicated by the convolution of wavelength shifts in the excitation and/or emission spectra of the dye with the transmission characteristics of the excitation/emission filters of

the microscope or other optical system. In our calcium measurements in neurosecretory terminals of the mouse neurohypophysis (Muschol and Salzberg, 1998), the fluorescence quenching of magnesium green by caffeine was nearly compensated by the improved optical transmission due to the increase in Stokes shift. In contrast, mag-fura-2 yielded a nearly twofold increase in the amplitude of the fluorescence transients in the presence of caffeine, despite its more than threefold higher K_d^{Cf} (see Table 1). Even ratiometric measurements are not free of these artefacts. Recall the calibration equation for ratiometric dyes (Grynkiewicz et al., 1985):

$$[\text{Ca}^{2+}] = K_d^{\text{Ca}} \left(\frac{R - R_{\min}}{R_{\max} - R} \right) \left(\frac{\epsilon_f}{\epsilon_b} \right) \quad (3)$$

where $R_{\min} = \epsilon_f(\lambda_1)/\epsilon_f(\lambda_2)$, $R_{\max} = \epsilon_b(\lambda_1)/\epsilon_b(\lambda_2)$, and the molar fluorescence ratio ϵ_f/ϵ_b is evaluated at either of the two measurement wavelengths λ_1 or λ_2 (ϵ corresponds to the proportionality coefficient (S) defined by Grynkiewicz, 1985). All three of these calibration constants change in the presence of caffeine. For the data in Fig. 1 A with $\lambda_1 = 374$ nm and $\lambda_2 = 332$ nm, the ratios before and after application of 30 mM caffeine are $R_{\min} = 1.95$ versus 2.0, $R_{\max} = 0.07$ versus 0.12, and $\epsilon_f/\epsilon_b(\lambda_1) = 15.5$ versus 8.3.

Assessing and eliminating caffeine effects

Of the four ion indicator dyes evaluated, all exhibit large changes in their fluorescence properties in response to millimolar concentrations of caffeine. This direct effect can seriously distort physiological measurements of caffeine-induced changes in intracellular calcium levels. No simple strategy, applicable to all dyes, for eliminating this artifact appears possible.

Based on our experience with the four dyes in this study, ratiometric dyes provide the best means for detecting and eliminating the direct effect of caffeine on calcium measurements. First, notice that the shift in the isosbestic point of mag-fura-2 is modest (<5 nm). Changes in fluorescence intensity at that wavelength are, therefore, nearly independent of [Ca²⁺]_i and are a reliable sign of the presence of caffeine artifacts. Furthermore, caffeine-induced changes in calcium affinity appear to be modest. In situ determination of the new calibration constants R_{\min} , R_{\max} , and ϵ_f/ϵ_b in the presence of caffeine would, in principle, permit compensation for the direct changes in fluorescence ratios resulting from caffeine or other drugs. A more comprehensive correction appears possible with indo-1, a dye for which it is possible to calculate an emission ratio. In contrast to the dyes in this study, O'Neill et al. (1990) reported that caffeine quenches indo-1 fluorescence emission uniformly, i.e., independent of both emission wavelength and calcium concentration. This enabled them to dissect fluorescence changes in ventricular myocytes into contributions due to calcium or caffeine and, in turn, to monitor the intracellular concentration of caffeine.

Beyond caffeine

As suspected earlier (Best and Abramcheck, 1985) and illustrated here with theobromine, methylxanthines other than caffeine affect dye fluorescence (Donoso et al., 1994). Direct drug-dye interactions resulting in changes in dye properties (absorption, quantum yield) are not likely to be confined to xanthines. The results of our hydrophobic substitutions of water with dioxane suggest hydrophobicity as the key mechanism responsible for the observed fluorescence changes. If this interpretation is correct, it implicates a much wider range of reagents and dyes as candidates for such interactions. In the end, it is the relative affinity of the drug for its physiological target(s) compared with that for the calcium indicator that determines the importance of drug-dye interactions for the measurements. Using ratio-metric dyes and monitoring dye response to drug application at the isosbestic point should alert the investigator to such artifacts. At the same time, the enhancement or quenching of dye fluorescence resulting from hydrophobic drug-dye interactions might prove a useful research tool in a variety of circumstances.

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