Does the Use of DM-Nitrophen, Nitr-5, or Diazo-2 Interfere with the Measurement of Indo-1 Fluorescence?

Robert W. Hadley, Mark S. Kirby, W. J. Lederer, and Joseph P. Y. Kao
Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201 and Department of Pharmacology, University of Kentucky College of Medicine, Lexington, Kentucky 40536

ABSTRACT Emission spectra of the photolabile Ca^{2+} chelators DM-nitrophen, nitr-5, and diazo-2 were studied alone, and in the presence of indo-1, to investigate potential interactions that would make the simultaneous manipulation and ratiometric measurement of the intracellular Ca^{2+} concentration difficult. Neither diazo-2 nor its photoproduct were found to be significantly fluorescent, and consequently concentrations of diazo-2 up to 20 times that of indo-1 did not distort the emission spectra of indo-1. DM-nitrophen was scarcely fluorescent, but its fluorescence did increase upon photolysis. In contrast to diazo-2 and DM-nitrophen, nitr-5 itself was found to be quite fluorescent, and this fluorescence was significantly increased upon photolysis. Thus, combined use of nitr-5 and indo-1 poses the most difficulty. The emission spectra of all the investigated compounds were used to define experimental conditions and calibration procedures that make possible simultaneous measurement and manipulation of the intracellular Ca2+ concentration.

INTRODUCTION

One of the most important recent technical advances in the study of the role of intracellular calcium ions is the development of photolabile Ca^{2+} chelators such as nitr-5 (Adams et al., 1988), diazo-2 (Adams et al., 1989), and DM-nitrophen (Kaplan and Ellis-Davies, 1988). The affinity of each compound for Ca^{2+} is altered upon exposure to ultraviolet light. Introduction of these agents into cells allows the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to be suddenly increased or decreased by a pulse of light. These properties have proven exceedingly useful in the study of disparate areas such as synaptic transmission (Zucker and Haydon, 1988; Delaney and Zucker, 1990), long-term potentiation (Malenka et al., 1992), Ca^{2+} channel modulation (Morad et al., 1988; Gurney et al., 1989; Hadley and Lederer, 1991), Na^+/Ca^{2+} exchange (Niggli and Lederer, 1991; Kofuji et al., 1992), and excitation-contraction coupling (Valdeolmillos et al., 1989; Niggli and Lederer, 1990). However, a principal limitation of these studies has been that the information about the photoinduced alterations in $[Ca^{2+}]_i$ has almost always been qualitative. The alterations in $[Ca^{2+}]_i$ have been followed through estimates based on the known affinities of the chelators and their photoproducts (Zucker and Haydon, 1988), by comparison with known changes in reference solutions (Morad et al., 1988; Gurney et al., 1989), by contractile changes in cell length (Valdeolmillos et al., 1989; Niggli and Lederer, 1990), and with nonratiometric Ca^{2+} indicators (Kao et al., 1989). The combined use of ratiometric Ca^{2+} indicators such as fura-2 and indo-1 with these chelators has been attempted only rarely (Delaney and Zucker, 1990; Marrion et al., 1991). In part, this may be due to the increased technical difficulty of the experiments. However, it could also be attributed to concern about unintended photolysis of these chelators by the ultraviolet light used with the ratiometric indicators, and to concern about possible interactions between the photolabile Ca^{2+} chelators and fluorescent Ca^{2+} indicators, such as indo-1.

There are several possible problems that can arise from simultaneous use of two compounds that have prominent optical properties. First, it is possible that the photolabile Ca^{2+} chelators might screen, or absorb, either the excitation light meant to excite indo-1, or the fluorescent light given off from indo-1. Second, the photolabile Ca^{2+} chelators might have significant fluorescence on their own. Thus, the photolabile Ca^{2+} chelators have significant potential to either reduce or distort the Ca^{2+} signal arising from indicators such as indo-1. A third problem is that the chelators are converted into their photoproducts in the course of the experiment, and the photoproducts may have considerably different optical properties (e.g., fluorescence) when compared with the starting material. We therefore thought it was essential to undertake a careful evaluation of the optical properties and possible interactions of the photolabile Ca^{2+} indicators and indo-1. An abstract describing these results has been published previously (Hadley et al., 1993).

METHODS

Indo-1, DM-nitrophen, nitr-5, and diazo-2 were dissolved in different concentrations and combinations in a buffer containing 100 mM KCl, 10 mM 4-morpholinepropanesulfonic acid, and 1 mM EGTA (pH 7.2). The buffer was kept at room temperature (22°C). Emission spectra of each solution were obtained in both 0-Ca and hi-Ca conditions. No $CaCl_2$ was added to the 0-Ca solutions, but for the hi-Ca solutions, $CaCl_2$ was added until it was 0.5 mM in excess of that required to saturate all of the Ca^{2+} buffers. Optical measurements were made in a Spex CM1T10I fluorometer, usually using an optical quartz microcell (Hellma, Jamaica, NY) with a path length of 100 μm. We have also done additional experiments using a microcell with a 10-μm path length, and these experiments will be noted in the figure legend. The total volume of the 100-μm microcell was 27 μl, whereas the volume of the 10-μm microcell was 3 μl.

Received for publication 4 March 1993 and in final form 15 September 1993.

Address reprint requests to Dr. W.J. Lederer, Department of Physiology, University of Maryland, School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201.

0006-3495/93/12/2537/10 $2.00

The emission spectra were obtained using an excitation wavelength of 355 nm. We chose this excitation wavelength because it is close to the optimum for stimulating indo-1 fluorescence. Fluorescence was measured every 2 nm between 370 and 570 nm; emission was integrated for 1 s for each measurement. Fluorescence was measured using front-face emission collection. The slit width was set so that the band pass for 50% transmission was 0.37–0.74 nm.

When desired, the photolabile Ca^{2+} chelators were converted to their photoproducts by exposure to an ultraviolet lamp (Ultra-violet Products, San Gabriel, CA). The output of the lamp was filtered to yield a broad UV band centered at approximately 360 nm. During the course of photolysis, absorbance spectra of the chelator solutions were measured periodically in a spectrophotometer. Photolysis was judged complete when the absorbance stabilized at a new level. The photoproducts were then diluted into the final buffer as required.

5-Hydroxyacetyl-5′-methyl-BAPTA (BAPTA: 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), the minor product generated during diazo-2 photolysis, can be obtained quantitatively by treating diazo-2 with dilute mineral acid (Adams et al., 1989). A pure sample of 5-hydroxyacetyl-5′-methyl-BAPTA was prepared by the reaction of 1 mg of the tetrapotassium salt of diazo-2 in 132 μl 50% aqueous ethanol with 70 mM HCl for 2 h at room temperature. The reaction mixture was lyophilized to remove solvent and excess HCl from the product. A 10 mM stock solution of the lyophilized product was adjusted to neutrality with KOH.

RESULTS

Fig. 1 shows emission spectra obtained from solutions containing 50 μM indo-1 and either no $CaCl_2$ and 1 mM EGTA (0-Ca), or with enough $CaCl_2$ added so that it was 0.5 mM in excess of that required to saturate all Ca^{2+} buffers (hi-Ca). The emission spectra were obtained using an excitation wavelength of 355 nm. Fluorescence was measured between 370 and 570 nm. The measurements were made in an optical microcell with a path length of 100 μm. This path length was chosen because it was not too far removed from path lengths of physiological interest, but yet permitted exact reproducibility.

The emission properties of indo-1 are essentially similar to those described previously(Grynkiewicz et al., 1985). Peak fluorescence is at 472 nm under Ca^{2+}-free conditions, but shifts to 416 nm in the presence of a saturating concentration of Ca^{2+} ions. These data are the reference to which all the rest of the data in the study will be compared.

Our purpose in undertaking this study was to look at the spectroscopic properties of the photolabile Ca^{2+} chelators by themselves and in combination with indo-1, to ascertain whether combining the techniques of Ca^{2+} photorelease and ratiometric $[Ca^{2+}]$ measurement would distort the $[Ca^{2+}]$ measurements. Perhaps the most likely problem to occur with the combined use of photolabile Ca^{2+} chelators and indo-1 is that the chelators could contribute enough fluorescence on their own to distort the fluorescence signal arising from indo-1. Fig. 2 examines this possibility with regard to DM-nitrophen. Emission spectra are plotted for 1 mM DM-nitrophen under four conditions: in the 0-Ca and hi-Ca solutions, and with intact DM-nitrophen and completely photolyzed DM-nitrophen. It can be seen that unphotolyzed DM-nitrophen is not very fluorescent; nor is the fluorescence very sensitive to the presence of Ca^{2+} ions. Peak fluorescence occurred at 418 nm in the high Ca^{2+} solution. However, it is clear from Fig. 2 that the DM-nitrophen photoproducts are much more fluorescent than the parent compound. Peak fluorescence occurred at 432 nm for photolyzed DM-nitrophen (hi-Ca), which is not very different from intact DM-nitrophen. Photolyzed DM-nitrophen was also insensitive to the presence of 0.5 mM excess $CaCl_2$.

Fig. 3 shows the effect different concentrations of intact or photolyzed DM-nitrophen have on the emission spectra of 50 μM indo-1. Panels *A* and *B* show the effect of unphotolyzed DM-nitrophen, whereas panels *C* and *D* demonstrate the effect of photolyzed DM-nitrophen. In each case, five emission spectra are shown, as DM-nitrophen was present at 0, 0.1, 0.25, 0.5, or 1 mM concentrations.

The most prominent effect adding intact or photolyzed DM-nitrophen had on the indo-1 emission spectra was to reduce peak fluorescence. This suppressive effect was weak with intact DM-nitrophen and stronger with photolyzed DM-nitrophen. The suppressive effect was seen across the entire emission range, so that it essentially scaled the indo-1 emis-

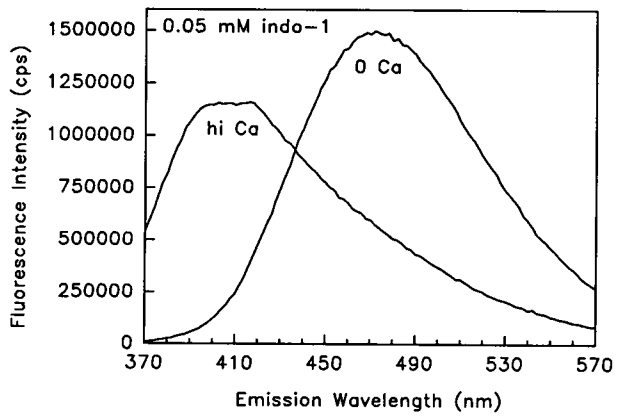

FIGURE 1 Emission spectra of indo-1. The emission spectra of 50 μM indo-1 were obtained under Ca^{2+}-free conditions (0-Ca), and in the presence of saturating Ca^{2+} (hi-Ca). Other experimental details were as described in Methods.

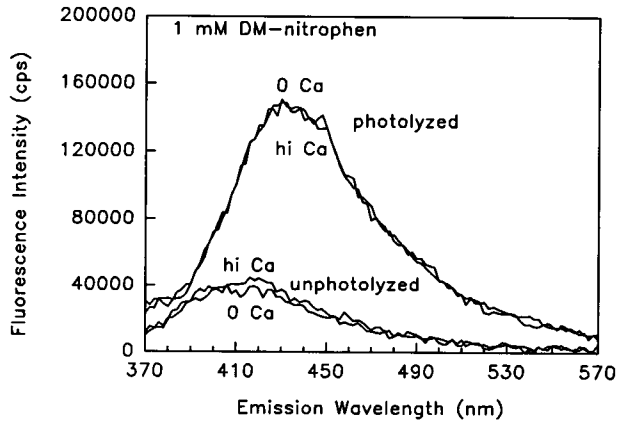

FIGURE 2 Fluorescence of DM-nitrophen and its photoproducts. Emission spectra are shown for 1 mM DM-nitrophen, both in 0-Ca and hi-Ca buffer, and with intact DM-nitrophen and completely photolyzed DM-nitrophen. Emission spectra were measured as described in Methods.

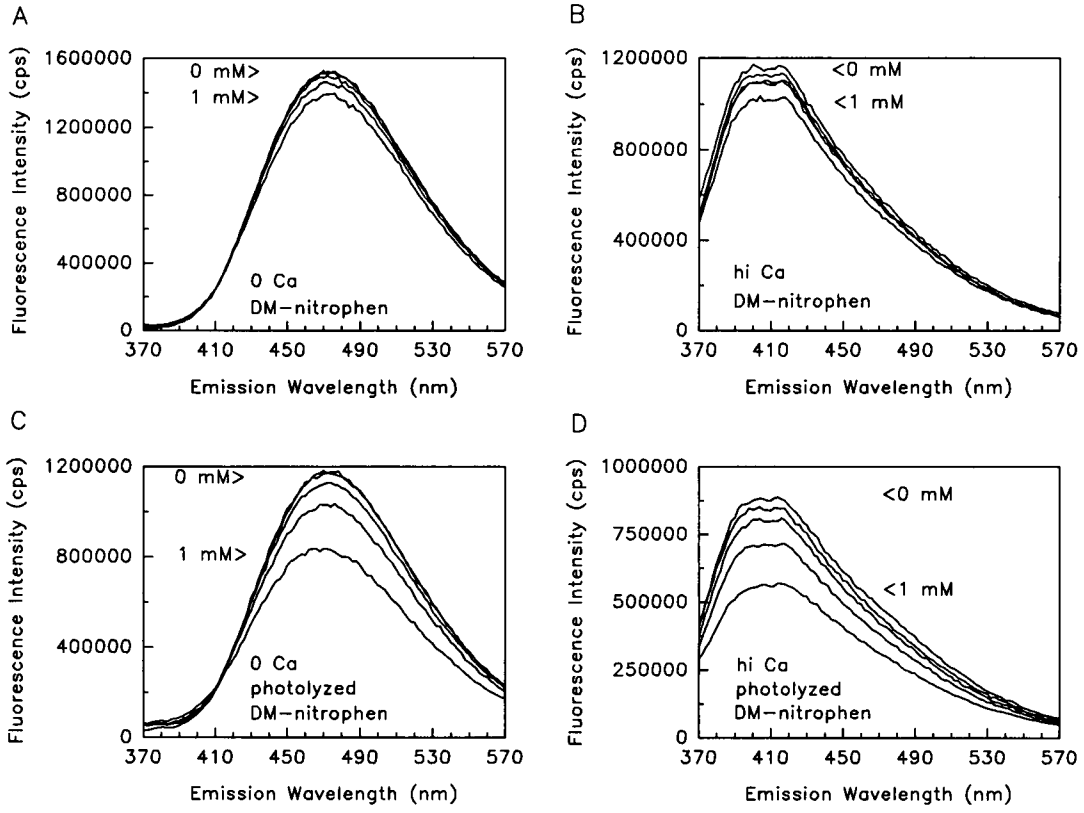

FIGURE 3 Emission spectra of indo-1 in the presence of different concentrations of DM-nitrophen or its photoproducts. In all the experiments shown, the buffer contained 50 μM indo-1 and 0, 0.1, 0.25, 0.5, or 1 mM DM-nitrophen or photolyzed DM-nitrophen. The curves obtained with DM-nitrophen are shown in panels *A* (0-Ca) and *B* (hi-Ca). The curves obtained with photolyzed DM-nitrophen are shown in panels *C* (0-Ca) and *D* (hi-Ca).

sion spectra. Furthermore, the suppressive effect was concentration-dependent, so that the curves with the highest peak fluorescence had 0 mM chelator, whereas the curves with the lowest peak had 1 mM chelator. The meaning and significance of this suppressive effect will be examined in more detail for the case of diazo-2, in which it is especially prominent.

It can be seen from Fig. 3, *A* and *B*, that the presence of DM-nitrophen in concentrations up to 20 times that of indo-1 does not distort the shape of the fluorescent indicator's emission spectrum. The same is true for photolyzed DM-nitrophen in hi-Ca solution (Fig. 3 *D*). However, photolyzed DM-nitrophen does cause a small distortion of the indo-1 emission spectra in 0-Ca solution. Indo-1 fluorescence is much less intense in 0-Ca solution in the range around 400 nm, which is close to the peak fluorescence of photolyzed DM-nitrophen. Therefore, the relative contribution of the DM-nitrophen photoproducts to measured indo-1 fluorescence would be maximal around 400 nm in 0-Ca solution. Nevertheless, the effect is slight, and only seen at the highest concentrations of photolyzed DM-nitrophen, where the emission curves can be seen to cross. Thus, it would be desirable to limit the generation of DM-nitrophen photoproducts during combined Ca^{2+} photorelease and measurement.

The second photolabile Ca^{2+} chelator that we examined was nitr-5, and the emission spectra for 1 mM nitr-5 and its photoproduct is shown in Fig. 4. It is obvious that both intact

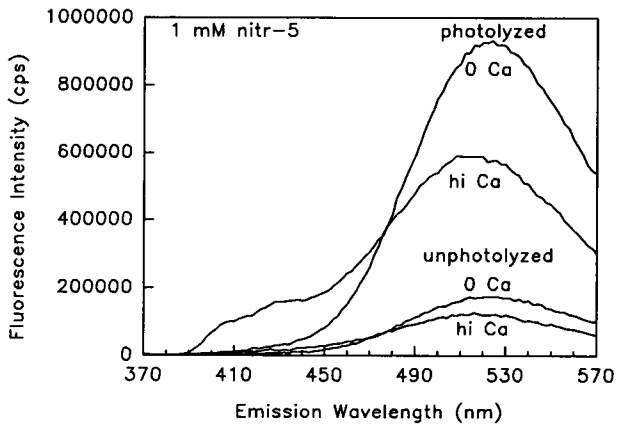

FIGURE 4 Fluorescence of nitr-5 and its photoproducts. Emission spectra are shown for 1 mM nitr-5, both in 0-Ca and hi-Ca buffer, and with intact nitr-5 and completely photolyzed nitr-5.

and photolyzed nitr-5 are considerably brighter than their DM-nitrophen counterparts, thus posing more of a problem. Both intact and photolyzed nitr-5 are most fluorescent in 0-Ca solution, with emission peaks at 524 nm. There is an additional complication in that the 0-Ca and hi-Ca curves cross over around 476 nm, so that emission in the 400–450-nm range is actually more prominent in hi-Ca solution.

The emission spectra of 50 μM indo-1 and different concentrations of intact and photolyzed nitr-5 were examined,

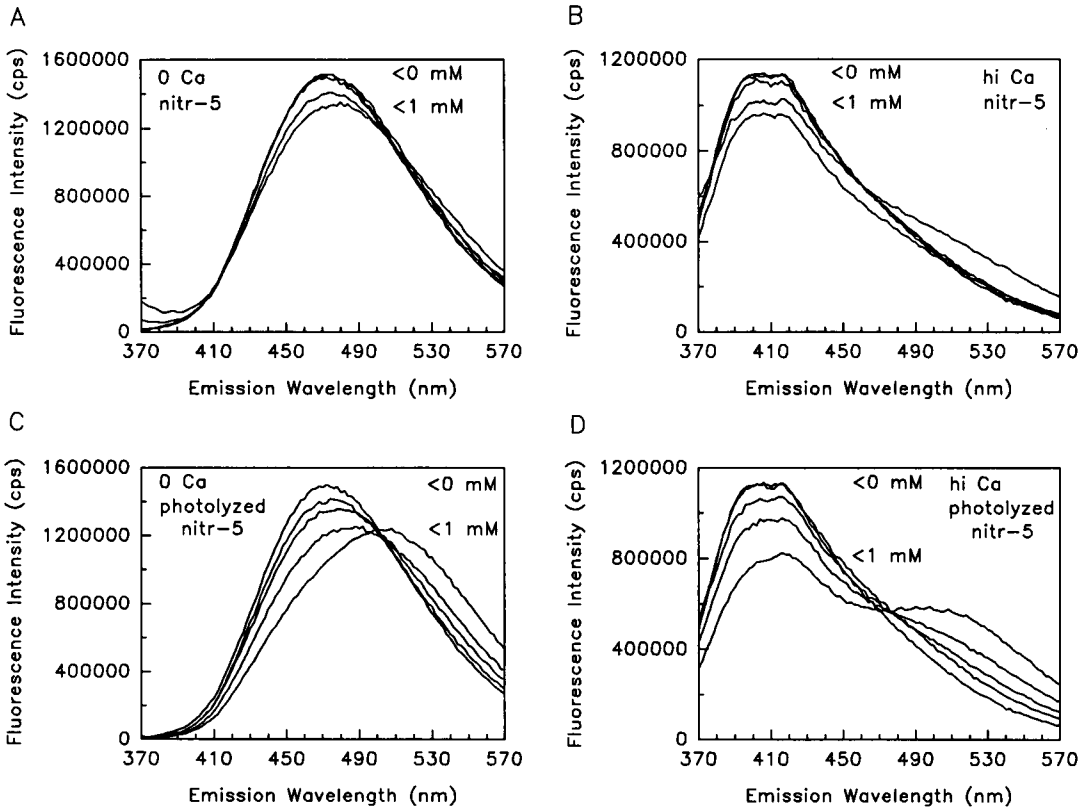

FIGURE 5 Emission spectra of indo-1 in the presence of different concentrations of intact or photolyzed nitr-5. The buffer contained 50 μM indo-1 and 0, 0.1, 0.25, 0.5, or 1 mM nitr-5 (panels *A* and *B*) or photolyzed nitr-5 (panel *C* and *D*).

and the results are shown in Fig. 5. Once again, both the intact and photolyzed Ca^{2+} chelator produce a concentration-dependent decrease in peak indo-1 fluorescence. However, in stark contrast to what was seen with DM-nitrophen, nitr-5 and its photoproduct are clearly capable of distorting the shapes of the indo-1 emission spectra. As could be predicted from the results in Fig. 4, this distortion consists of extra fluorescence seen in the emission range around 500 nm. This effect is accentuated both in the hi-Ca solution, as indo-1 fluorescence shifts toward the short-wavelength part of the spectrum, and with photolyzed nitr-5, as it is more fluorescent than the parent compound.

Diazo-2 is a particularly interesting photolabile Ca^{2+} chelator because its affinity for Ca^{2+} ions actually increases upon exposure to ultraviolet light. We studied the feasibility of combining diazo-2 with indo-1 in Figs. 6 and 7. Fig. 6 plots the emission spectra of unphotolyzed and photolyzed diazo-2. Both diazo-2 and its photoproduct are scarcely fluorescent, with their signal being just discernible from background.

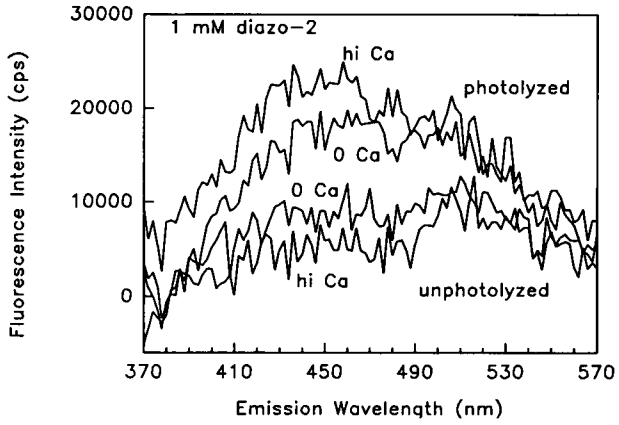

FIGURE 6 Fluorescence of diazo-2 and its photoproduct. Emission spectra are shown for 1 mM diazo-2, both in 0-Ca and hi-Ca buffer, and with intact diazo-2 and completely photolyzed diazo-2. Note the low level of fluorescence.

Fig. 7 examines how diazo-2 and its photoproduct affect indo-1 fluorescence in the same way that DM-nitrophen and nitr-5 were examined. It would be expected that neither intact nor photolyzed diazo-2 would distort the shape of the indo-1 emission spectra, as indo-1 is so much more fluorescent than the other compounds. This proves to be the case. However, diazo-2 diminished indo-1 fluorescence across the entire emission range more effectively than DM-nitrophen or nitr-5. Photolyzed diazo-2 scales down the indo-1 emission spectra in a very similar fashion. This scaling effect would seem to be a less serious perturbance of the indo-1 signal than the extra fluorescence seen with nitr-5, because the standard ratio procedure that is used to calibrate the indo-1 signal should reduce or eliminate the effect. However, the loss of signal is certainly unwanted, and two questions remain. First, what is the likely mecha-

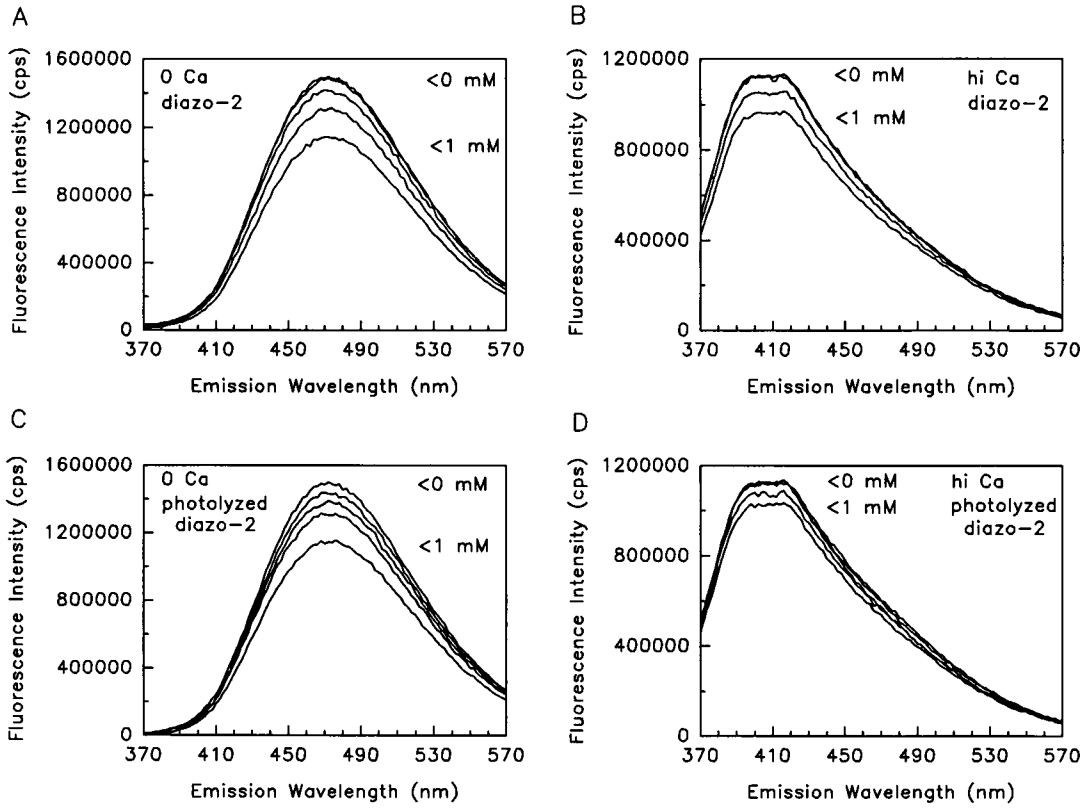

FIGURE 7 Emission spectra of indo-1 in the presence of different concentrations of intact or photolyzed diazo-2. The buffer contained 50 μM indo-1 and 0, 0.1, 0.25, 0.5, or 1 mM diazo-2 (panels *A* and *B*) or photolyzed diazo-2 (panels *C* and *D*).

nism behind this scaling effect? Second, what significance is the scaling effect likely to have in a living cell?

Perhaps the most likely mechanism for this scaling effect is screening. Diazo-2, and the other photolabile Ca^{2+} chelators as well, could be absorbing either some of the excitation light meant to excite indo-1 molecules, or the emitted fluorescence from indo-1. Because the reduction is uniform across the emission range of 370–570 nm, but diazo-2 absorbance is not uniform over the same wavelength range (Adams et al., 1989), it is the excitation light that would have to be screened by diazo-2. This explanation seems reasonable, and we tested this idea in the experiment shown in Fig. 8. Quite simply, perturbances of the emission spectra that are dependent on absorbance, rather than fluorescence, would be strongly dependent on the path length. The longer the path length, the more likely it would be that photons would be intercepted by a diazo-2 molecule before reaching an indo-1 molecule. The emission spectra in Fig. 8 show that this is the case for diazo-2. Both panels *A* and *B* show emission spectra obtained in 0-Ca solutions, with 50 μM indo-1 alone, or in combination with 1 mM diazo-2. However, the data in panel *A* were obtained in a microcell with a 100-μm path length, and the data in panel *B* were obtained with a 10-μm path length. Peak indo-1 fluorescence at 472 nm was reduced 24% in panel *A* and 5% in panel *B*. This fits theoretical predictions as well, as calculations using Beer's law and the known extinction coefficient (Adams et al., 1989) predict that 1 mM diazo-2 would diminish indo-1 fluorescence by 20% and 2% in the 100-μm and 10-μm microcells, respectively. Additional experiments with DM-nitrophen and nitr-5 (not shown) revealed that they had almost no suppressive effect at a path length of 10 μm, in agreement with what would be expected from Beer's law. This idea is further supported by the data shown in Fig. 8 *C*, in which the concentration dependence with which diazo-2 suppresses indo-1 fluorescence is plotted for both 0-Ca and hi-Ca solutions. The suppression has a linear dependence on the diazo-2 concentration, which is what is predicted by Beer's law. Plainly, screening of excitation light by diazo-2, DM-nitrophen, and nitr-5 seems to be the most likely explanation for the scaling effect.

However, the photoproducts appear to behave quite differently. Fig. 9, *A* and *B*, demonstrates that 1 mM photolyzed diazo-2 also suppresses indo-1 fluorescence, but shortening the path length from 100 μm to 10 μm only reduced the suppression from 26% to 24%. Similar results were also obtained with photolyzed DM-nitrophen and nitr-5. Thus, these photoproducts attenuate indo-1 fluorescence through another mechanism besides screening.

This new mechanism of fluorescence suppression can be studied most easily in the case of photolyzed diazo-2, because it is the least fluorescent of the photoproducts. Several characteristics of the suppression can be identified. First, it is independent of path length. Second, the suppression is independent of emission wavelength, as is clear from Fig. 9, *A* and *B*. Third, the suppression has a linear concentration dependence, as shown in Fig. 9 *C*. Addi-

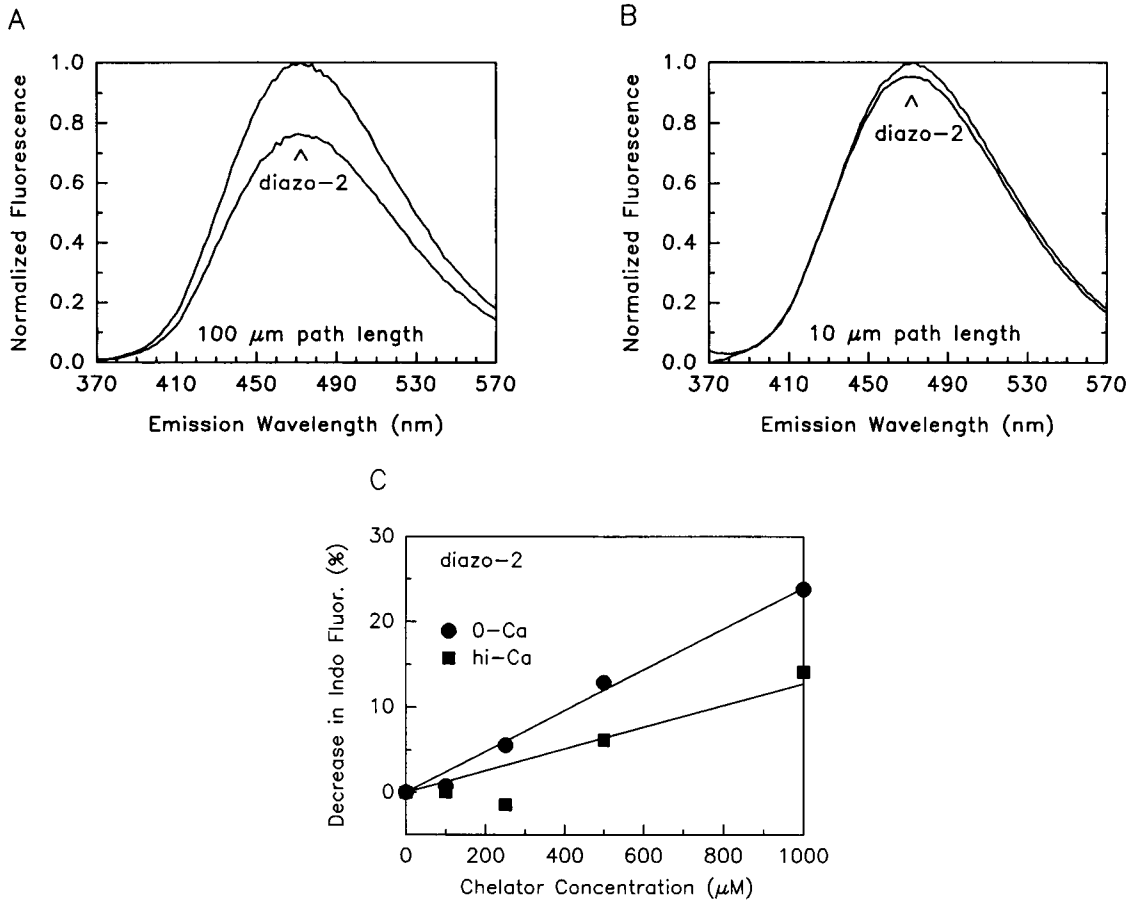

FIGURE 8 Attenuation of indo-1 fluorescence by 1 mM diazo-2. Panel *A* plots the emission spectra of 50 μM indo-1 in the presence and absence of 1 mM diazo-2, when using an optical microcell with a 100-μm path length. The data were obtained in Ca^{2+}-free conditions and were taken from Fig. 7. Panel *B* shows data obtained during an identical experiment, except that a microcell with a 10-μm path length was used. Panel *C* plots the concentration-dependent attenuation of indo-1 fluorescence by free and Ca^{2+}-bound diazo-2, using a 100-μm path length. The plot was obtained by calculating the attenuation present in the dataset for Fig. 7, *A* and *B*, after subtracting DZ fluorescence. The lines were fit by linear regression.

tional studies of photolyzed DM-nitrophen and nitr-5 (not shown) indicated that they also shared these properties, although the data were noisy due to the presence of added fluorescence.

The simplest interpretation of these data is that photolyzed diazo-2 is attenuating the indo-1 fluorescence through collisional quenching. Photolyzed diazo-2 cannot be interfering with the excitation light, because suppression is independent of path length. The interference cannot be at the level of emitted fluorescence, because the suppression is independent of both path length and emission wavelength. A direct physical interaction with indo-1 is the most likely possibility remaining. This suggestion is consistent with the linear concentration dependence of the suppression (Fig. 9 *C*), because collisional quenching should increase linearly with concentration. Preliminary experiments suggested that the main diazo-2 photoproduct, 5-carboxymethyl-5′-methyl-BAPTA, was not responsible for the quenching. However, 5-hydroxyacetyl-5′-methyl-BAPTA, an expected side product of photolysis, did quench indo-1 fluorescence.

DISCUSSION

Summary and comparison with the literature

Table 1 summarizes the principal observations of this study. The relative fluorescence intensity (compared with indo-1) and peak emission wavelength are given for DM-nitrophen, nitr-5, and diazo-2, as well as their respective photoproducts, for both the 0-Ca and hi-Ca solutions. In addition, the table lists the amount of attenuation of indo-1 fluorescence expected from a 1 mM concentration of each compound for both 0-Ca and hi-Ca solutions, when using a 100-μm path length. The numbers were obtained from plots such as shown for DZ and DZ-P in Figs. 8 *C* and 9 *C*.

Recently, Zucker (Zucker, 1992) published the results of a study that had similar objectives. Specifically, that study reported on possible interference by DM-nitrophen, nitr-5, and their respective photoproducts on $[Ca^{2+}]$ measurements made with the Ca^{2+} indicators fluo-3 and fura-2. However, there were several reasons why we didn't feel that this report provided enough information for us to safely incorporate

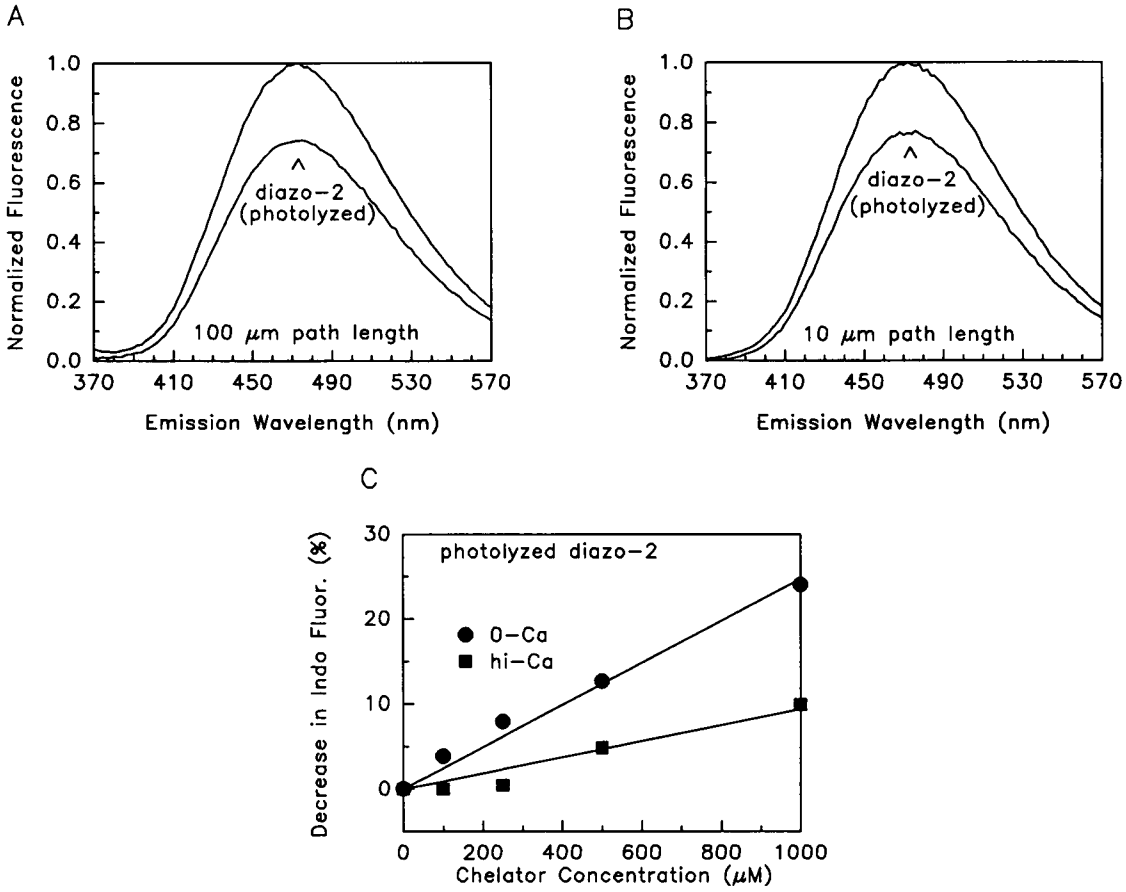

FIGURE 9 Attenuation of indo-1 fluorescence by 1 mM photolyzed diazo-2. Panels *A* and *B* show the attenuation in 100-μm and 10-μm microcells, respectively. Panel *C* plots the concentration-dependent attenuation of indo-1 fluorescence by free and Ca^{2+}-bound photolyzed diazo-2. Other details are the same as for Fig. 8.

$[Ca^{2+}]_i$ measurements into the Ca^{2+} photorelease experiments we have been doing on single heart cells and cell lines (Hadley and Lederer, 1991; Kofuji et al., 1992). First, Zucker's study did not examine two of the compounds we were interested in: diazo-2 and indo-1. Second, we were concerned that Zucker's data might overestimate the problems that we would see in our experiments. Zucker used very high concentrations of DM-nitrophen and nitr-5, up to 100 times the concentration of Ca^{2+} indicator. Our own studies only explored interactions up to a concentration ratio of 20:1 (chelator/indicator). Third, we felt that Zucker's recommendations for the calibration of the fluorescent Ca^{2+} indicators would not work well for the types of experiments we had in mind. Zucker suggested construction of multiple Ca^{2+} calibration curves, with different sets of calibration solutions corresponding to the experimental conditions before and after photolysis. It therefore becomes necessary to have an accurate knowledge of the concentrations of indicator, chelator, and photolyzed chelator throughout the time course of the experiment. We felt that this approach was not likely to prove accurate for whole-cell patch-clamp experiments, because it would involve calculations that would include several estimated factors. It was also likely to prove technically arduous, inasmuch as a single experiment might involve several photolysis events, and the degree of photolysis might be expected to vary from experiment to experiment.

The results of the previous study (Zucker, 1992) and the present study can be directly compared with regard to the spectroscopic properties of DM-nitrophen and nitr-5. Both studies agree that DM-nitrophen is poorly fluorescent, but nitr-5 fluorescence is considerable. The two studies are also in agreement that DM-nitrophen photoproducts are much brighter than DM-nitrophen itself. However, the studies disagree on two points. First, Zucker reported that Ca^{2+} enhanced the fluorescence of photolyzed DM-nitrophen. We did not see this, but this is almost certainly attributable to the fact that we only added 0.5 mM excess $CaCl_2$, whereas Zucker added 5 mM $CaCl_2$. Because the K_d value for photolyzed DM-nitrophen has been reported to be approximately 3 mM (Kaplan and Ellis-Davies, 1988), both reports are probably accurate. Our "hi-Ca" solution contained only 0.5 mM excess $CaCl_2$, because we thought that higher concentrations were of no physiological interest, and could not be measured by indo-1 anyway. Second, we have reported that photolysis enhanced nitr-5 fluorescence, which Zucker did not see. However, the methods differed considerably. We

TABLE 1 Summary of fluorescence data

	Peak F (nm)		Peak F (%)		Attenuation (%)	
	0-Ca	hi-Ca	0-Ca	hi-Ca	0-Ca	hi-Ca
Indo-1	472	416	100	100	—	—
DM	408	418	0.13	0.19	11.5	14.7
DM-P	432	432	0.50	0.64	37.0	41.4
N5	524	516	0.58	0.55	13.3	13.1
N5-P	524	508	3.1	2.5	42.3	40.0
DZ	510	516	0.042	0.055	25.3	11.6
DZ-P	460	458	0.066	0.11	22.1	9.4

F is an abbreviation for fluorescence. DM, N5, and DZ are abbreviations for DM-nitrophen, nitr-5, and diazo-2, respectively. DM-P, N5-P, and DZ-P are the abbreviations for the completely photolyzed compounds. The second and third columns given the wavelength at which peak fluorescence occurred for each compound, whereas the fourth and fifth columns give the relative intensity of that peak fluorescence, normalized to the peak fluorescence of indo-1 under the same condition. The last two columns give the attenuation of 50 μM indo-1 fluorescence expected for 1 mM of each chelator.

completely photolyzed nitr-5 to study its properties in isolation, whereas Zucker studied partially photolyzed nitr-5 to mimic what occurs during an actual experiment. Thus, the amount and types of compounds present during the measurements actually differed considerably. Therefore, it would seem worthwhile to undertake further studies of the effect of photolysis on nitr-5 fluorescence, only the additional studies should be done in an actual whole-cell patch-clamp experiment. This would allow a definitive determination of which dataset is more applicable to single-cell experiments.

Calibration of indo-1 in the presence of photolabile Ca^{2+} chelators

We have decided that the simplest and most useful approach to simultaneously manipulate and measure $[Ca^{2+}]$ is to define experimental conditions that cause minimal perturbance of the normal indo-1 calibration. Therefore, the first question that has to be answered is how much error can be tolerated? There probably is no absolute answer to this question, because the degree of accuracy required will vary from study to study. However, a maximal error of 10% might be a good starting point. This seems quite reasonable, given that there are already significant limits on the precision with which $[Ca^{2+}]$ can be measured with fluorescent indicators such as fura-2 and indo-1 (Baylor and Hollingworth, 1988; Hove-Madison and Bers, 1992). However, it is also important to note that the error produced by adding photolabile Ca^{2+} chelators to an indo-1 solution will not be constant with $[Ca^{2+}]$. For instance, peak nitr-5 fluorescence occurs around 520 nm. Therefore, the indo-1 ratios will be most sensitive to induced error at a high $[Ca^{2+}]$, where the indo-1 signal around 500 nm is minimized. However, the error induced by DM-nitrophen and its photoproducts will be aggravated by a low $[Ca^{2+}]$, because peak fluorescence of these species occurs at 430 nm.

It was therefore necessary for us to calculate the error induced by different concentrations of both intact and photolyzed DM-nitrophen, nitr-5, and diazo-2 at high and low $[Ca^{2+}]$. The factor that is of predominant concern is the distortion of the indo-1 signal by added fluorescence from the chelators. However, attenuation of the indo-1 signal through screening or quenching mechanisms also needs to be taken into account. Use of the ratioing technique and shorter path lengths will reduce these errors, but quenching is independent of path length, and both screening (Fig. 8 *C*) and quenching (Fig. 9 *C*) can be Ca^{2+}-dependent. The error calculations were therefore made using the dataset collected with a path length of 100 μm, because the dataset will include contributions from added fluorescence, quenching, and screening. The calculations were made for a $[Ca^{2+}]$ of either 0.1 or 1 μM, because these concentrations span the physiological $[Ca^{2+}]_i$ range that is of most interest in our experiments. Each computation consisted of first calculating the concentration of free and Ca^{2+}-bound species of indo-1 and photolabile Ca^{2+} chelator from their known K_d and the $[Ca^{2+}]$. The fluorescence that each species contributes could then be calculated for various emission wavelengths by referring back to the appropriate emission spectra (Figs. 1, 2, 4, and 6). The attenuation of indo-1 fluorescence could then be calculated by referring back to the values given in Table 1. Thus emission ratios could be calculated for indo-1 alone, and after addition of different concentrations of each photolabile Ca^{2+} chelator. The percentage error in the $[Ca^{2+}]$ estimate induced by a given compound could then be calculated by using the standard equation (Grynkiewicz et al., 1985)

$$Ratio = R_{min} + \frac{R_{max} - R_{min}}{1 + K^*/[Ca]}$$

R_{min} and R_{max} are the minimum and maximum ratio of indo-1 fluorescence, and were found to be 0.098 and 3.08 for a 400/500 nm ratio. K^* is equal to the K_d for Ca^{2+} binding to indo-1 multiplied by the fluorescence ratio at 500 nm of the 0-Ca to the hi-Ca solution, and was 802 nM. Different values of R_{min}, R_{max}, and K^* were calculated for each emission ratio.

The calculations were made for two emission ratios for each photolabile Ca^{2+} chelator. These consisted of the standard 400/500 nm emission ratio, and another designed to minimize the fluorescence contribution from the photoproducts. In the case of DM-nitrophen, the 400/500 nm ratio was already close to optimal.

The effect that different concentrations of all three photolabile Ca^{2+} chelators and their photoproducts have on $[Ca^{2+}]$ measurements made with indo-1 are listed in Table 2. Basically, use of diazo-2 or its photoproduct causes minimal error in concentrations up to 20 times that of indo-1. Use of a 420/480 nm ratio only gives a modest improvement, but might be useful for other reasons (see below). DM-nitrophen itself causes some error, but its photoproduct causes more. DM-nitrophen can be used at almost 20 times the concentration of indo-1 before the 10% error limit is reached, whereas only half as much photoproduct can be tolerated.

TABLE 2 Effect of photolabile Ca^{2+} chelators on $[Ca^{2+}]$ measurements

[chelator] (μM)	100	100	250	250	500	500	1000	1000
[indo] (μM)	50	50	50	50	50	50	50	50
[Ca] (μM)	0.1	1	0.1	1	0.1	1	0.1	1
DM (400/500)	101	100	103	101	106	103	113	108
DM-P (400/500)	101	99	105	99	110	99	127	98
DZ (400/500)	100	100	100	99	100	98	100	96
DZ (420/480)	100	100	101	100	101	100	103	100
DZ-P (400/500)	100	99	100	99	100	97	101	95
DZ-P (420/480)	100	100	101	100	102	99	104	98
N5 (400/500)	98	95	95	88	91	79	83	63
N5 (400/436)	100	99	100	98	100	96	100	92
N5-P (400/500)	89	75	75	52	56	32	25	14
N5-P (400/436)	99	97	99	93	97	86	92	70

The table shows the effect on $[Ca^{2+}]$ measurements of the addition of different concentrations of several compounds at two different true $[Ca^{2+}]$ concentrations, and with different emission ratios. The data are normalized to what would be observed in the presence of indo-1 alone. (Values given are percentages. 100 indicates no effect.)

$[Ca^{2+}]$ measurements with indo-1 are quite sensitive to nitr-5 and its photoproduct. The nitr-5 concentration can only be five times that of indo-1 before significant error occurs, and the photoproduct concentration has to be kept very low. The data in Table 2 also demonstrate that considerable improvement can be seen if $[Ca^{2+}]$ measurements are made using 400/436 nm ratios rather than 400/500 nm, so as to make the measurements further away from the peak of nitr-5 fluorescence.

Conclusions

This report has established conditions under which $[Ca^{2+}]$ can be accurately measured with indo-1 during the use of the photolabile Ca^{2+} chelators DM-nitrophen, nitr-5, and diazo-2. Several points deserve additional comment. First, although use of the 420/480 nm emission ratio for diazo-2 provides only slight improvement in the error calculations, it may avoid a problem inherent with the 400/500 nm ratio. Adams et al. (1989) have reported absorbance spectra for diazo-2 that indicate there should be significant absorbance of indo-1 fluorescence at 400 nm, but not at 420 nm. This could be another source of error, although examination of Fig. 7 revealed no evidence that this occurred in these studies. However, the problem could be completely avoided by using a short path length, or the 420/480 nm ratio.

Another point worthy of amplification is the choice of emission ratios for use with nitr-5. At first glance, the 400/436 nm ratio appears to be the superior choice. However, use of this ratio is not problem-free. First, R_{min} and R_{max} are 0.098 and 3.08 for the 400/500 nm ratio, but are only 0.133 and 1.22 for the 400/436 nm ratio. This almost fourfold compression of the difference between R_{min} and R_{max} will diminish the resolution of the calibration procedure. Second, the 400/436 nm ratio may be an ideal choice for cuvette experiments, in which more fluorescence is available for measurement. However, such experiments would tax the standard instrumentation used for single-cell experiments on a fluorescence microscope. Such experiments would require dichroic mirrors and interference filters (or monochrometers) with sharp band pass properties that would eliminate most of the fluorescence from a very limited source. Many investigators may therefore be interested in making a tradeoff between experimental error and technical difficulties. In order to be flexible, Table 3 provides detailed information about the fluorescence properties of indo-1, nitr-5, and photolyzed nitr-5 at several wavelengths between 400 and 500 nm. Together with the attenuation data in Table 1, it would be possible to make error calculations for any emission ratios which might be thought to be a good compromise.

A third point that should be mentioned is that although this study has concentrated on minimizing errors in $[Ca^{2+}]$ measurements through varying the emission wavelengths and the concentration of the photolabile Ca^{2+} chelators and indo-1, it should also be possible to optimize the excitation wavelength to minimize contributed fluorescence from the chelators. However, this does not appear to be a productive approach. First, diazo-2 is already scarcely fluorescent. Second, examination of the absorbance spectra of photolyzed DM-nitrophen (Kaplan and Ellis-Davies, 1988) shows that

TABLE 3 Relative fluorescence of indo-1 and nitr-5

	Indo-1 (%)		N5 (%)		N5-P (%)	
λ (nm)	0 Ca	hi Ca	0 Ca	hi Ca	0 Ca	hi Ca
400	8.12	76.3	0.0188	0.0177	0.0222	0.226
410	16.3	76.4	0.0147	0.0275	0.0416	0.332
420	31.8	75.4	0.0190	0.0409	0.0697	0.422
430	49.8	67.2	0.0275	0.0494	0.101	0.529
440	68.6	59.1	0.0421	0.0770	0.160	0.538
450	84.5	51.7	0.0594	0.0930	0.271	0.623
460	94.4	45.2	0.0956	0.139	0.507	0.811
470	99.0	39.9	0.174	0.182	0.865	1.02
480	97.0	33.9	0.262	0.238	1.38	1.33
490	92.6	28.9	0.377	0.310	1.96	1.60
500	83.2	24.8	0.484	0.373	2.54	1.80

The table gives the relative fluorescence of free and Ca^{2+}-bound indo-1, N5, and N5-P at several emission wavelengths (λ) between 400 and 500 nm. The data have been normalized relative to the fluorescence of free indo-1 at 472 nm.

the absorbance decreases at wavelengths longer than 355 nm. However, this property cannot be used to minimize chelator fluorescence, because the move to a longer excitation wavelength would also diminish indo-1 fluorescence (Grynkiewicz et al., 1985). Similar frustrations are also apparent in trying to optimize the excitation wavelength for use with nitr-5. The absorbance of free photolyzed nitr-5 increases at wavelengths longer than 355 nm, whereas the absorbance of Ca^{2+}-bound photolyzed nitr-5 increases at wavelengths shorter than 355 nm (Adams et al., 1988).

Finally, it should be mentioned that although this report has rigorously examined the spectroscopic interactions of indo-1 and the photolabile Ca^{2+} chelators, additional problems could arise during biological experiments. These include photobleaching of indo-1 by the ultraviolet light intended to photolyze the chelator compounds, and unintended photolysis of the chelators by the ultraviolet light intended to excite indo-1. In practice, we have found that neither of these problems pose a grave difficulty (Kirby et al., in press).

This research was supported by National Institutes of Health grants GM46956 (J.P.Y.K.) and HL36974 (W.J.L.). This work was also supported in part by a Grant-in-Aid from the American Heart Association (R.W.H.), and with funds contributed in part by the American Heart Association, Maryland Affiliate.

REFERENCES

Adams, S. R., J. P. Y. Kao, G. Grynkiewicz, A. Minta, and R. Y. Tsien. 1988. Biologically useful chelators that release Ca^{2+} upon illumination. *J. Am. Chem. Soc.* 110:3212–3220.

Adams, S. R., J. P. Y. Kao, and R. Y. Tsien. 1989. Biologically useful chelators that take up Ca^{2+} upon illumination. *J. Am. Chem. Soc.* 111: 7957–7968.

Baylor, S. M., and S. Hollingworth. 1988. Fura-2 calcium transients in frog skeletal muscle fibres. *J. Physiol.* 403:151–192.

Delaney, K. R., and R. S. Zucker. 1990. Calcium released by photolysis of DM-nitrophen stimulates transmitter release at squid giant synapse. *J. Physiol.* 426:473–498.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Gurney, A. M., P. Charnet, J. M. Pye, and J. Nargeot. 1989. Augmentation of cardiac calcium current by flash photolysis of intracellular caged-Ca^{2+} molecules. *Nature.* 341:65–68.

Hadley, R. W., M. S. Kirby, W. J. Lederer, and J. P. Y. Kao. 1993. Do interactions between photolabile Ca chelators and indo-1 prevent simultaneous measurement and manipulation of $[Ca_i]$? *Biophys. J.* 64:A40.

Hadley, R. W., and W. J. Lederer. 1991. Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *J. Physiol.* 444:257–268.

Hove-Madsen, L., and D. M. Bers. 1992. Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophys. J.* 63:89–97.

Kao, J. P. Y., A. T. Harootunian, and R. Y. Tsien. 1989. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J. Biol. Chem.* 264:8179–8184.

Kaplan, J. H., and G. C. R. Ellis-Davies. 1988. Photolabile chelators for the rapid photorelease of divalent cations. *Proc. Natl. Acad. Sci. USA.* 85: 6571–6575.

Kirby, M. S., R. W. Hadley, and W. J. Lederer. Measurement of $[Ca^{2+}]$; using indo-1 during simultaneous flash photolysis to release Ca^{2+} from DM-nitrophen. *Pfluegers Arch.* In press.

Kofuji, P., R. W. Hadley, R. Kieval, W. J. Lederer, and D. H. Schulze. 1992. Expression of the Na/Ca exchanger in diverse tissues: a study using the cloned human cardiac Na/Ca exchanger. *Am. J. Physiol.* 263:C1241–C1249.

Malenka, R. C., B. Lancaster, and R. S. Zucker. 1992. Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron.* 9:121–128.

Marrion, N. V., R. S. Zucker, S. J. Marsh, and P. R. Adams. 1991. Modulation of M-current by intracellular Ca^{2+}. *Neuron.* 6:533–545.

Morad, M., N. W. Davies, J. H. Kaplan, and H. D. Lux. 1988. Inactivation and block of calcium channels by photo-released Ca^{2+} in dorsal root ganglion neurons. *Science.* 241:842–844.

Niggli, E., and W. J. Lederer, 1990. Voltage-independent calcium release in heart muscle. *Science.* 250:565–568.

Niggli, E., and W. J. Lederer. 1991. Molecular operations of the sodium-calcium exchanger revealed by conformation currents. *Nature.* 349:621–624.

Valdeolmillos, M., S. C. O'Neill, G. L. Smith, and D. A. Eisner. 1989. Calcium-induced calcium release activates contraction in intact cardiac cells. *Pfluegers Arch.* 413:676–678.

Zucker, R. S. 1992. Effects of photolabile calcium chelators on fluorescent calcium indicators. *Cell Calcium.* 13:29–40.

Zucker, R. S., and P. G. Haydon. 1988. Membrane potential has no direct role in evoking neurotransmitter release. *Nature.* 335:360–362.