Photodegradation of indo-1 and its effect on apparent Ca^{2+} concentrations

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Background: Fluorescent indicators that show alterations in excitation and/or emission spectra in response to changes in $[Ca^{2+}]$ are widely used for quantitative cytosolic $[Ca^{2+}]$ measurements. There are several reports of changes in apparent $[Ca^{2+}]$ due only to illumination, however. These results have been attributed either to photodamage to the cells or to photodegradation of the indicator. Light-induced alteration in the behavior of the dye or cells would severely hamper the interpretation of experimental data. We examined this phenomenon in indo-1 loaded cells using confocal laser scanning microscopy.

Results: Illumination of indo-1 loaded GH3 cells leads to a decrease in apparent basal $[Ca^{2+}]$ and decreased peaks after depolarization with KCl. When cells were double loaded with indo-1 and fluo-3, the effect of UV illumination was noticed only with the former dye. UV irradiation of indo-1 in simple buffers caused overall photobleaching and conversion to a fluorescent but Ca^{2+}-insensitive species. The latter effect cannot be canceled by ratiometric calibration and is due to loss of carboxymethyl groups from the anilino nitrogens. This photodegradation was inhibited by extracellular administration of 10 to 100 μM Trolox, a water-soluble vitamin E analog.

Conclusions: Photodegradation processes like that observed for indo-1 are likely to be possible for all cation indicators that contain bis(carboxymethyl)anilino moieties, which include essentially all fluorescent indicators for Ca^{2+} and Mg^{2+} currently in biological use. If unrecognized, this photochemical dealkylation leads to an underestimation of the analyte concentrations depending on the intensity and duration of illumination. The problem can be avoided by including cell-permeant antioxidants such as Trolox in the bathing solution. The ultimate solution would be to redesign the indicators to minimize photodegradation in the absence of antioxidants.

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Introduction

Studies concerning regulation and interactions of second messengers are progressively being performed at the single-cell level. The development of Ca^{2+}-sensitive dyes like fura-2 and indo-1 has provided invaluable information on the mechanisms of cellular Ca^{2+} homeostasis [1]. The latest technical improvement in the study of Ca^{2+} homeostasis is confocal microscopy [2–4]. This technique has a higher spatial resolution than classical imaging instruments, so that subcellular changes of $[Ca^{2+}]$, like differences between cytoplasmic regions [2], cytoplasm versus nucleus [3] or the spatial complexity of Ca^{2+} waves [4], can be more easily resolved.

Until recently, most confocal laser scanning microscopes (CLSM) were equipped with visible wavelength lasers, typically having an excitation wavelength of 488 nm. Due to this limitation, confocal Ca^{2+} measurements were performed on cells loaded with visible wavelength dyes like fluo-3 or Fura-red [5]. A disadvantage of these dyes is that Ca^{2+} binding mainly changes their fluorescence intensity, instead of their peak emission wavelength, making calibration in terms of Ca^{2+} concentration difficult. Attempts to circumvent this problem using a combination of fluo-3 and Fura-red injected into cells have met with some success, giving a more quantitative fluorescence signal [5]. However, it is harder to apply this approach to the acetoxymethyl ester loading technique, since differences in loading efficiency, subcellular distribution and bleaching properties [2] are difficult to correct.

Recently, UV-laser compatible confocal microscopes have been developed, making the dye indo-1, which undergoes an emission shift upon Ca^{2+} binding, a superior probe for quantitative studies of Ca^{2+} homeostasis with CLSM. With indo-1, fluorescence data can be easily calibrated in terms of Ca^{2+} concentration by the standard ratiometric technique [6,7], which cancels out variations in the overall

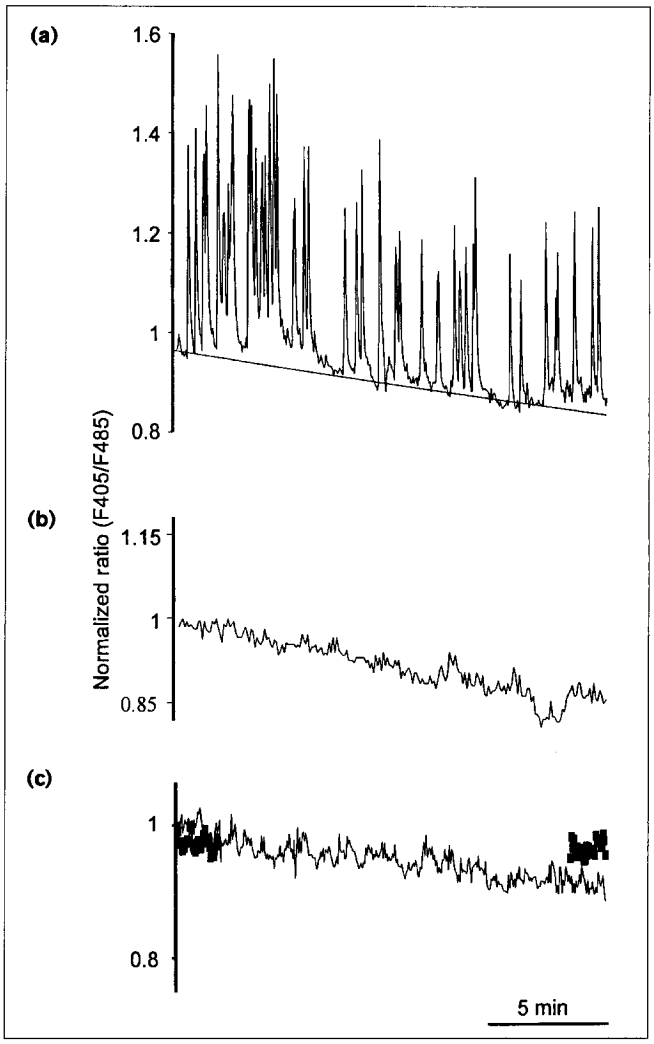
amount of fluorescent indicator. However, some problems have been encountered with this dye, in particular rapid bleaching and an apparent decrease of ratio values upon prolonged laser illumination [8]. Fura-2 has also been reported to be convertible to fluorescent forms that are Ca^{2+}-insensitive [9], resulting in decreasing ratio values. Such ratio changes can severely affect the reliability of the calibration. We have therefore investigated the behavior of indo-1 under UV illumination. It was found that UV light induces a change in the spectral properties of indo-1, with a reduction in ratio values at a constant Ca^{2+} concentration. Photodegradation thus seems to be a general problem for these dyes. A method for preventing this phenomenon in intact cells will be presented.

Results

Figure 1a shows the change in apparent $[Ca^{2+}]$, as revealed by indo-1 fluorescence emission ratio (405/485 nm), in a single GH3 cell continuously illuminated with the laser beam (351 nm). Repetitive spontaneous oscillations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were observed, whose amplitude tended to decrease with time. Similar Ca^{2+} oscillations have been previously observed by Schlegel *et al.* [10] in GH3 cells and by Malgaroli *et al.* [11] in primary cultures of pituitary cells, and have been attributed to spontaneous action potentials. We have also observed that the oscillations ceased immediately upon chelation of extracellular Ca^{2+} or addition of L-type Ca^{2+} channel blockers. About 60 % of the cells displayed spontaneous Ca^{2+} oscillations (n = 642). Not only did the amplitude of the spikes decline with time, but also the values of the downward deflections between the spikes showed a tendency to decrease in the course of the experiment, suggesting a decline of basal $[Ca^{2+}]$. This phenomenon can be better appreciated in a non-oscillating cell (Fig. 1b). The 405/485 ratio, set to 1 at the beginning of the experiment, was 0.85 after 10 min of illumination. A monoexponential curve fitting was performed on the baseline ratio values using the formula $F_t = F_0 e^{-ut}$, where u represents the monoexponential decay value [12]. Curve fitting was performed for a number of cells, and was found to be fairly constant for all cells within an experiment, leading to u values of $0.002 \pm 0.001\ s^{-1}$. The decrease in baseline fluorescence ratio was more pronounced in cells that had been illuminated with a higher laser power (data not shown). If cells are incubated under the same conditions for 10 min, but one group is protected from the exciting beam (by a mask) for most of the time, the reduction in 405/485 ratio occurs only in the cells continuously exposed to the laser light (Fig. 1c).

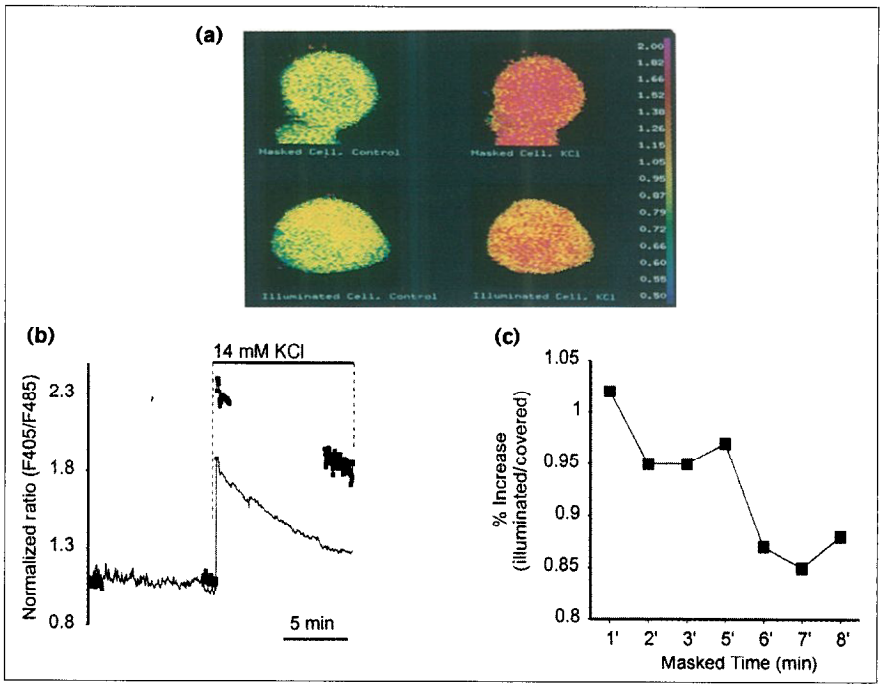
Once the cells have been heavily illuminated, their fluorescence ratios are abnormal, even when the $[Ca^{2+}]_i$ is increased, for example by depolarizing the membrane potential with high KCl. Figure 2a shows pseudocolor ratio images of two cells, at rest (left panel) and at the peak of the $[Ca^{2+}]_i$ increase induced by addition of 14 mM KCl (right panel). The lower cell was illuminated for 10 min before adding KCl, the upper one for the minimum time required to acquire the images shown in Figure 2. The difference in the peak amplitude is obvious. The ratio values of a number of cells (n = 13

Figure 1



Continuous illumination changes the apparent $[Ca^{2+}]_i$ in GH3 cells. Cells were loaded with 5 μM indo-1/AM for 30 min in the presence of 0.02 % Pluronic F-127 and 0.2 μM sulfinpyrazone to inhibit nonspecific anion exporters. After loading, cells were washed and allowed to equilibrate for another 15 min before the experiment was started. The normalized ratio of the fluorescences at 405 and 485 nm are presented. **(a)** GH3 cell showing spontaneous oscillations. Basal ratio values decreased by 10 % during the 10 min exposure time. Peak height during spontaneous oscillations decreased by 15 %. **(b)** Normalized ratio values of a GH3 cell not showing spontaneous oscillatory activity. The decrease in baseline ratio is better observed and was 15 % during 10 min for this particular cell. **(c)** Comparison of ratio values between a group of cells continuously illuminated (continuous line, n = 24) with that of a group that was protected from illumination by a mask for 8 min (blocks, n = 19). The decrease in ratio values was observed only in continuously illuminated cells.

Figure 2



Continuous illumination affects the increase in apparent $[Ca^{2+}]_i$ induced by depolarization. Loading conditions were the same as those described in Fig. 1. **(a)** Pseudocolor images of two GH3 cells. The top cell was covered by a mask for most of the time, the bottom cell was continuously illuminated. The picture on the left panel was taken at the beginning of the experiment. Both cells had the same starting ratio. The picture on the right panel was taken after 10 min illumination and 10 s after application of 14 mM KCl. **(b)** Comparison of KCl-induced increase in ratio of two populations of cells, one being illuminated continuously, the other being masked for 8 min. The KCl-induced increase was 23 % smaller in the continuously illuminated cells. **(c)** Kinetics of illumination-dependent decrease in the KCl response. Prior to application of 14 mM KCl half of the cells were continuously illuminated for 1 to 8 min, whereas half of the cells were masked for most of the time. The KCl-induced increase in ratio values of the masked cells was set to 100 %.

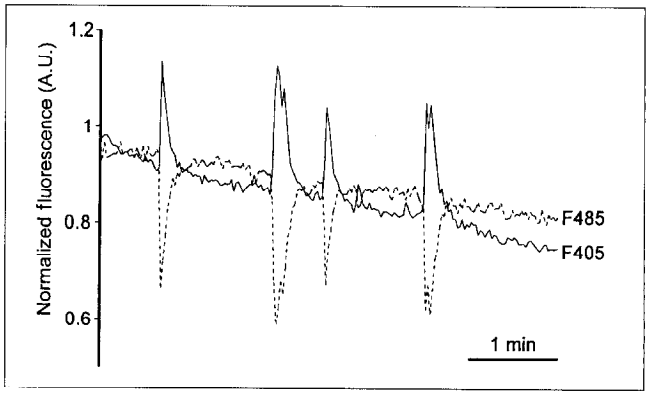
masked, n = 11 unmasked) of such experiments were averaged (Fig. 2b). The mean reduction in peak amplitude for this experiment was 23 %; similar results were obtained in six different trials. Figure 2c shows the decrease in peak amplitude for various illumination times. It can be seen that the reduction in mean peak amplitude depended on the duration of exposure to the laser beam prior to KCl application.

Figure 3 shows the kinetics of fluorescence emission at the two wavelengths analyzed individually for a cell continuously illuminated for 5 min. For simplicity, the fluorescence intensity at either wavelength was normalized to 100 % at the beginning of the experiment. Rather than showing an identical reduction, as expected for simple bleaching, the 405 trace dropped more rapidly than the 485 trace, explaining the decrease in the 405/485 ratio.

To determine whether the observed reduction in fluorescence emission ratio was due to a toxic effect on the cells of the UV illumination or an intrinsic characteristic of indo-1, we examined the effect of illumination on indo-1 trapped in the lumen of liposomes under the same conditions. The liposomes contained either Ca^{2+}-free medium (no Ca^{2+} and 5 mM EGTA) or high-Ca^{2+} medium (5 mM Ca^{2+}), plus 100 μM of the free acid form of indo-1 (Table 1). It can be seen that illumination induced a reduction in indo-1 emission ratio, both in liposomes containing Ca^{2+}-free and high-Ca^{2+} medium.

Direct evidence that the reduction in ratio seen for indo-1 is not attributable to cell damage by the UV light is provided in Figure 4. GH3 cells were double loaded with indo-1 and fluo-3. After exposing half of the cells to the UV laser for 10 min, the excitation wavelength was switched to 488 nm, and fluo-3 emission in the range 510 to 540 nm was captured. Application of KCl resulted in a fluorescence increase, which was kinetically and quantitatively indistinguishable in UV-exposed cells compared to masked cells (Fig. 4, n = 80).

Figure 3



The fluorescence emission intensity at F405 (solid line) decreases more rapidly than that at F485 (dashed lines) upon contiunous illumination. Conditions as in Fig. 1. The individual fluorescence intensities were normalized to 1 at the beginning of the experiment.

The simplest explanation of the above results is that photolysis of indo-1 does not simply bleach the fluorescence but also generates a new fluorescent compound whose emission spectrum resembles Ca^{2+}-free indo-1 and is relatively insensitive to Ca^{2+}. Accordingly, photolysis decreases the fluorescence emission ratio and simulates a decrease of $[Ca^{2+}]$. This surmise is confirmed in Figure 5. Figure 5a shows the photolysis of indo-1 in Ca^{2+}-free EGTA. Enough 365 nm illumination was delivered to bleach about 73 % of the apparent emission amplitude. However, most of the remaining fluorescence was no longer derived from unchanged indo-1, because addition of excess Ca^{2+} caused only a modest shift of the emission spectrum to shorter wavelengths. The resulting emission spectrum was much different from a scaled-down version of that of unphotolyzed indo-1 saturated with Ca^{2+}. Figure 5b shows the photolysis of indo-1 in excess Ca^{2+}. The main effect of illumination was simply to bleach the Ca^{2+} complex, not to shift the fluorescence back towards that of free indo-1. Therefore the formation of the Ca^{2+}-insensitive product emitting at 460 nm is less pronounced when the photolysis occurs at high $[Ca^{2+}]$.

Most mechanisms of dye photodestruction involve oxidation of the dye by reactive oxygen species such as singlet oxygen or superoxide. Photodestruction of the dye and photodynamic damage to cells can often be greatly ameliorated by removal of ambient O_2, addition of antioxidants, or both. Removal of O_2 is not usually an option for work on living mammalian cells. We therefore examined the effects of a number of antioxidants on indo-1 photodestruction both *in vitro* and, where possible, in live cells. Thiols such as 2-mercaptoethanol and glutathione were ineffective, at least at low millimolar concentrations. Ascorbic acid was quite effective *in vitro*, but not when applied extracellularly at concentrations of 50 or 500 μM. The most effective agent found was a water-soluble vitamin E analog, Trolox. Figure 6 compares photodegradation of indo-1 in Ca^{2+}-free EGTA with and without 500 μM Trolox. This high dose of antioxidant slowed the rate of overall bleaching by a factor of about

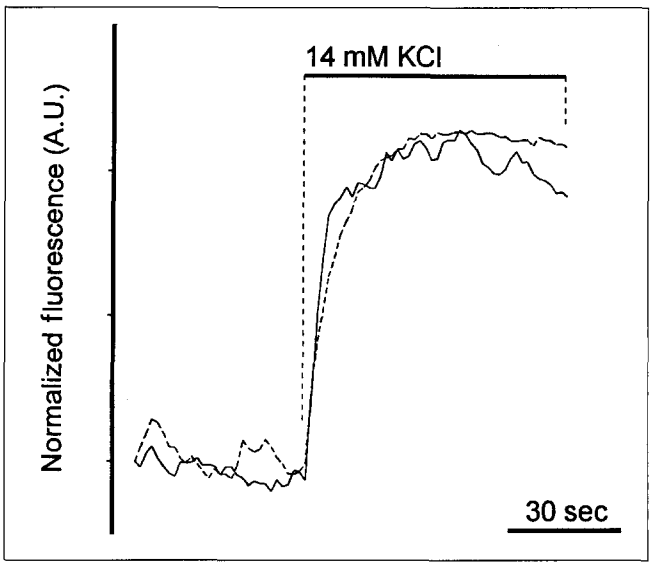
Table 1

Effect of UV illumination and protection by Trolox on indo-1 fluorescence trapped within liposomes.

	Ca^{2+}-free EGTA	5 mM Ca^{2+}
Control	21.4 ± 2.8	15.4 ± 3.2
10 μM Trolox	24.1 ± 1.6	15.9 ± 6.2
100 μM Trolox	9.7 ± 1.7[a]	13.9 ± 1.6

[a]$P < 0.05$ vs control, Ca^{2+}-free EGTA ($n = 24$)
Liposomes containing 100 μM indo-1 were prepared as described in the methods. The internal liposome medium contained either no added $CaCl_2$ but 5 mM EGTA instead, or 5 mM $CaCl_2$. The data are presented as % decrease in ratio values caused by 10 min illumination.

Figure 4



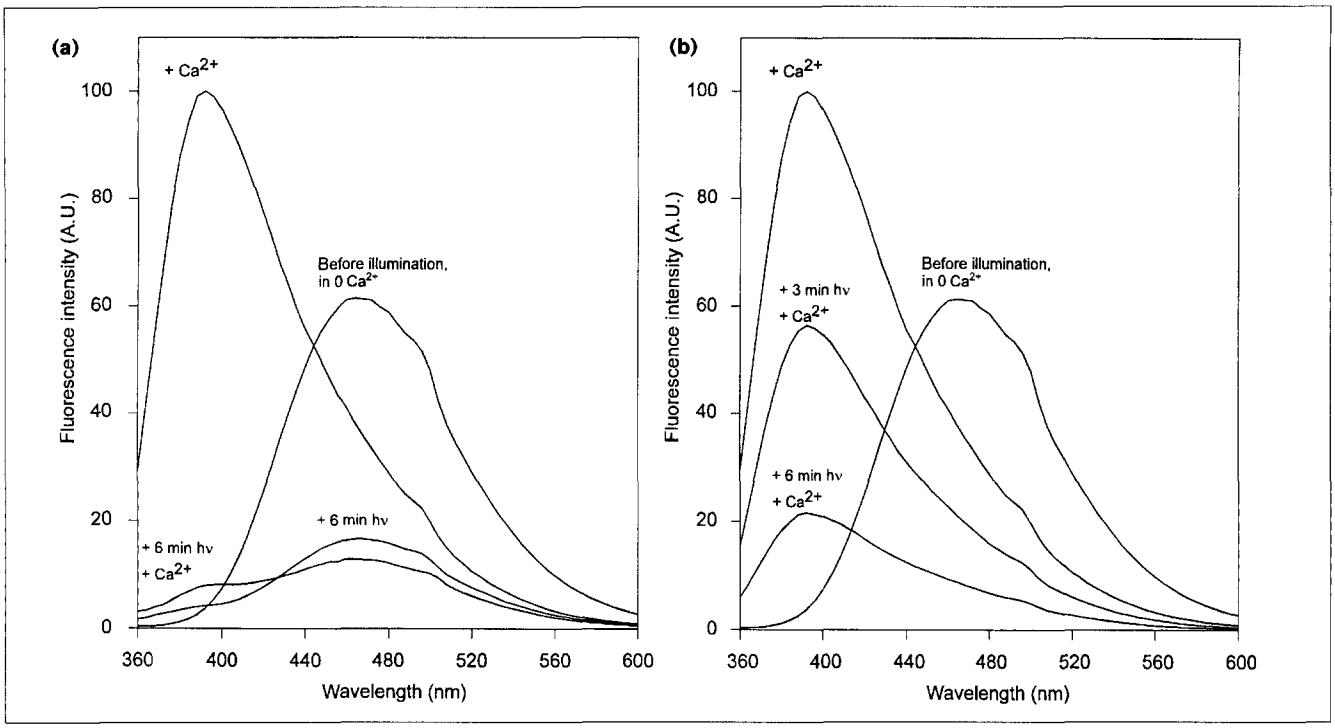
Prolonged UV illumination does not affect KCl induced fluo-3 intensity. Cells were double loaded with 5 μM indo-1/AM and 5 μM fluo-3/AM for 30 min. Prior to capturing the fluo-3 fluorescence, half of the cells were illuminated with the UV laser for 10 min using conditions similar to those of Figs 1,2. Fluo-3 fluorescence intensity of both UV-illuminated (continuous line) and non-illuminated cells (dashed line) was captured before and after addition of 14 mM KCl.

10. More importantly, subsequent addition of excess Ca^{2+} gave the correct spectrum for Ca^{2+}-bound indo-1, revealing that Trolox had essentially prevented the formation of the Ca^{2+}-insensitive form that depresses the emission ratio.

Figure 7a shows a comparison of KCl induced $[Ca^{2+}]_i$ peaks in experiments similar to that shown in Figure 3, but in cells preincubated with different concentrations of Trolox. Whereas a concentration of 0.1 μM was practically ineffective, in cells treated with 1 μM Trolox the peak height induced by KCl in cells continuously illuminated for 10 min was only 5 % lower than in masked cells. At Trolox concentrations above 10 μM, no difference between peak height or basal level was observed between the two groups of cells. Trolox also prevented the reduction in baseline ratio values. Figure 7b shows the preventive effect of 10 μM Trolox on the reduction in baseline ratio in a non-oscillating cell. Trolox also partially prevented the reduction in the 405/485 ratio values caused by illumination of indo-1 in liposomes, though higher concentrations were necessary (Table 1).

Finally, the effects of Trolox itself on Ca^{2+} signaling were examined. In 85 % of the cells, addition of 100 μM Trolox stopped spontaneous oscillations for 1–2 min, after which the oscillations reappeared with amplitude and frequency indistinguishable from those observed just before addition of the drug. The effect of 100 μM Trolox on a cell

Figure 5

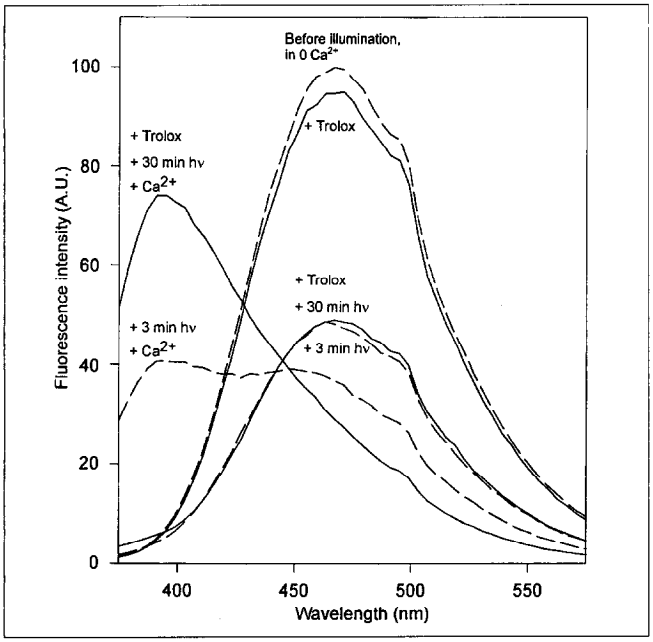


Photolysis generates a new fluorescent compound whose emission spectrum resembles Ca^{2+}-free indo-1 but which is relatively insensitive to Ca^{2+}. Emission spectra changes after 365 nm irradiation of 2 μM indo-1 in the absence **(a)** and presence **(b)** of Ca^{2+} are shown. (a) The emission spectrum of 2 μM indo-1 excited at 350 nm in 2 ml of 100 mM KCl, 10 mM Tris, 5 mM MOPS, 2 mM EGTA, pH 7.4 is shown before illumination (0 Ca). The less intense emission spectrum shows the same sample after irradiation for 6 min with intense 365 nm light (+ 6 min hν). Addition of a saturating amount of Ca^{2+} to the irradiated sample produced the emission spectrum labeled (+ 6 min hν + Ca^{2+}). The emission spectrum for an identical sample of indo-1 in the presence of saturating Ca^{2+} which was not irradiated is shown for comparison (+ Ca^{2+}). (b) The emission spectrum of 2 μM indo-1 excited at 350 nm in 2 ml of 100 mM KCl, 10 mM Tris, 5 mM MOPS, 2 mM EGTA, pH 7.4 is shown before illumination (0 Ca). The emission spectrum labeled (+ Ca^{2+}) was obtained by the addition of $CaCl_2$ to produce a free [Ca^{2+}] of 2 mM. Irradiation of the Ca^{2+} saturated sample with 365 nm light for 3 min produced the emission curve labeled (+ 3 min hν + Ca^{2+}). Irradiation of the same sample for another 3 min produced the curve labeled (+ 6 min hν + Ca^{2+}).

showing very large and frequent oscillations is shown in Figure 7c. At concentrations of 10 μM this effect of Trolox was observed only in 23% of the cells. The drug had no appreciable effect on non-oscillating cells.

Although practical biological uses of indo-1 and Trolox do not require knowledge of the photodegradation mechanism or products, such information would be of chemical interest and invaluable for future redesign of the indicator to minimize the need for antioxidant additives. Figure 8 presents the most likely reaction pathways, based on the known photochemistry of related indoles and phenylglycines and the preliminary observation (L.R.M., unpublished data) that removal of O_2 greatly retards the overall bleaching but, surprisingly, not the formation of the Ca^{2+}-insensitive species. Reactive oxygen species such as singlet oxygen are known to attack the 2,3-double bond of indoles to give dioxetanes (Fig. 8, intermediate A), which spontaneously decompose to products such as 2-formylanilides (product B) [13] and 4H-3,1-benzoxazin-4-ones (product C) [14]. Both products would probably be non-fluorescent, or at least much less fluorescent than indo-1 at its standard wavelengths of excitation. Even without O_2 or oxygen species present, indoles tend to eject solvated electrons in their excited state, leaving behind a cation radical (intermediate D) [15]. If the electron-rich BAPTA moiety is not coordinating Ca^{2+}, it might donate an electron to the indole cation radical, which would restore the indole to the ground state but form a new cation radical on the aniline nitrogen (intermediate E). Such cation radicals are known to decarboxylate readily [16], which would result in a dye (product F) with a normal indo-1-like fluorophore but little or no ability to bind Ca^{2+}. An entire carboxymethyl side chain can also be lost [17]; such a product (G) would also fluoresce like indo-1 but be crippled with respect to chelating properties. Prior binding of Ca^{2+} to the BAPTA portion of the molecule would prevent formation of the cation radical at the aniline nitrogen and inhibit formation of the postulated Ca^{2+}-insensitive species. Trolox scavenges singlet oxygen [18]

Figure 6

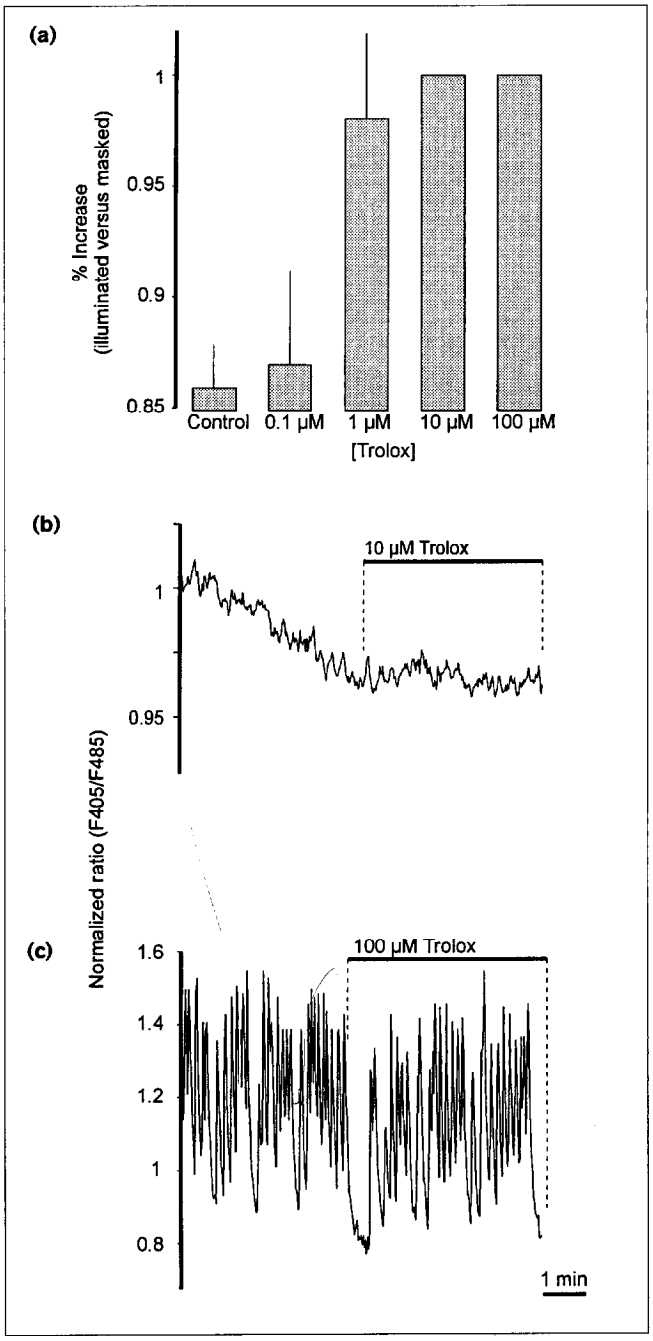


Trolox inhibits the bleaching and photodegradation of indo-1. The effect of 500 μM Trolox on the changes in the emission spectra of indo-1 is shown after irradiation with 365 nm light in the presence of Ca^{2+}. The dashed lines show the emission spectra of 2 μM indo-1 excited at 350 nm in 2 ml of 100 mM KCl, 10 mM Tris, 5 mM MOPS, 2 mM EGTA, pH 7.4, before illumination (0 Ca), after 3 min irradiation with 365 nm light (+ 3 min hν) and after a saturating amount of Ca^{2+} was added to the irradiated sample (+ 3 min hν + Ca^{2+}). The solid lines show the emission spectra of 2 μM indo-1 excited at 350 nm in 2 ml of 100 mM KCl, 10 mM Tris, 5 mM MOPS, 2 mM EGTA, 500 μM Trolox, pH 7.4 (+ Trolox), after the Trolox-containing solution was irradiated for 30 min with 365 nm light of the same intensity as before(+ Trolox + 30 min hν) and after a saturating amount of Ca^{2+} was added to the irradiated sample (+ Trolox + 30 min hν + Ca^{2+}).

and should competitively inhibit oxidation of indo-1 to nonfluorescent species. Trolox would presumably also re-reduce indo-1 cation radicals back to indo-1, intercepting them before they could dismember their Ca^{2+}-binding sites. In the process, Trolox would itself be oxidized to a radical, but such hindered phenoxy radicals are relatively unreactive and innocuous. Trolox analogously protects cells from a considerable variety of free-radical-mediated cytotoxic insults [19].

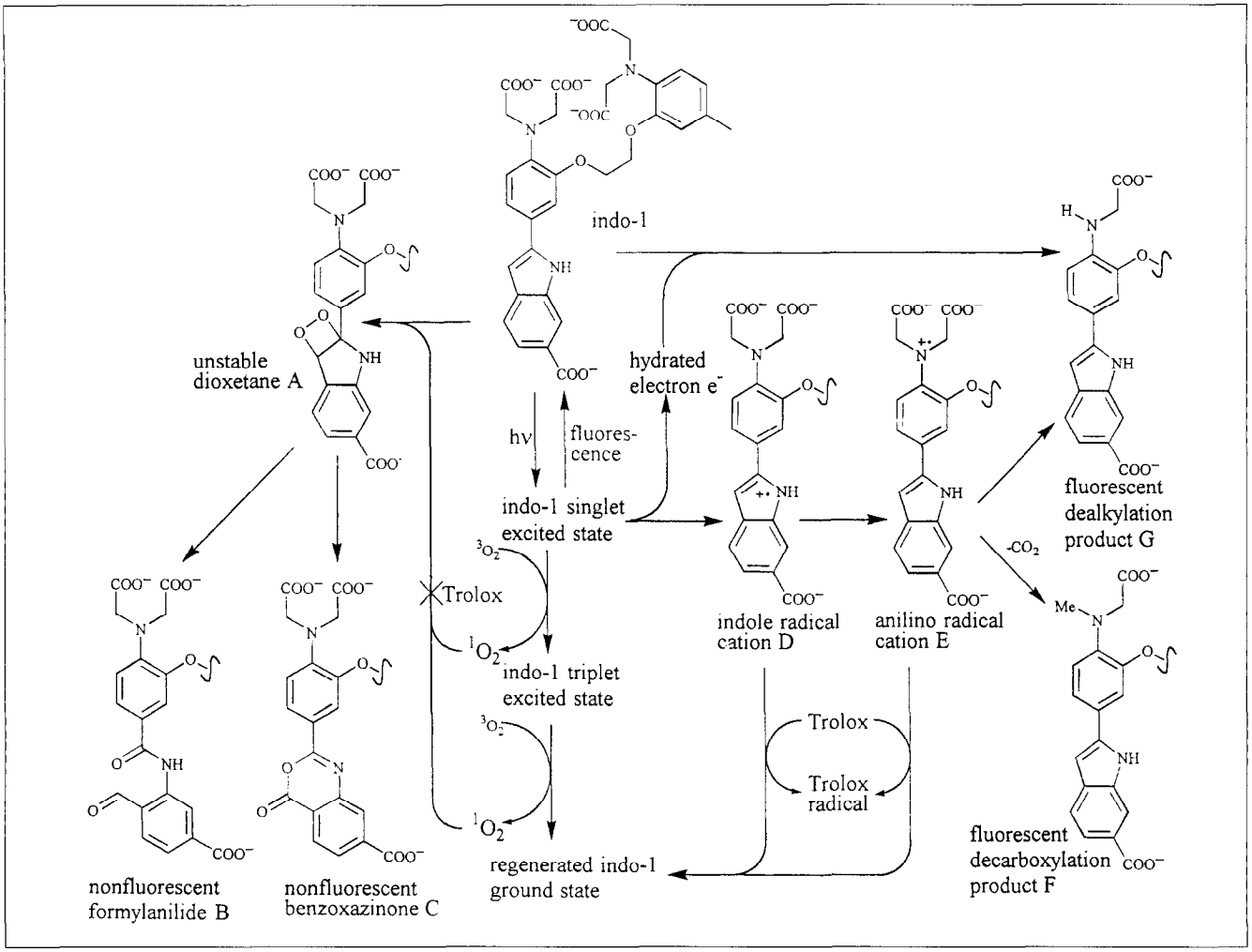
Mass spectrometry is probably the most sensitive and definitive technique to determine the identity of the fluorescent Ca^{2+}-insensitive product. Therefore indo-1 at 0.5 mM in 20 mM diisopropylethylamine bicarbonate, pH 7.8, was photolyzed at 365 nm under argon to about 20 % conversion. The lack of Ca^{2+} and O_2 was intended to channel the photochemistry towards formation of the fluorescent Ca^{2+}-insensitive product, which is biologically more important and interesting than the nonfluorescent products. The low percentage conversion should have

Figure 7



Effects of Trolox on $[Ca^{2+}]_i$ in GH3 cells. Conditions as in Figure 1. **(a)** Half of the cells were continuously illuminated for 10 min; the other half were masked for 8 min. 14 mM KCl was applied and KCl-induced ratio increases of the two populations were compared as described in Fig. 2c. Different concentrations of Trolox were included in the bath solution, as indicated. The results presented were obtained from the same batch of cells. The number of cells tested were: control, illuminated n = 24, masked n = 26; 0.1 μM Trolox, illuminated n = 19, masked n = 18; 1 μM Trolox, illuminated n = 27, masked n = 25; 10 μM Trolox, illuminated n = 23, masked n = 29; 100 mM Trolox, illuminated n = 27, masked n = 25. Similar results were obtained in three independent trials. **(b)** Effect of 10 μM Trolox on the decrease in baseline ratio. **(c)** Effect of 100 μM Trolox on spontaneous Ca^{2+} oscillations.

Figure 8



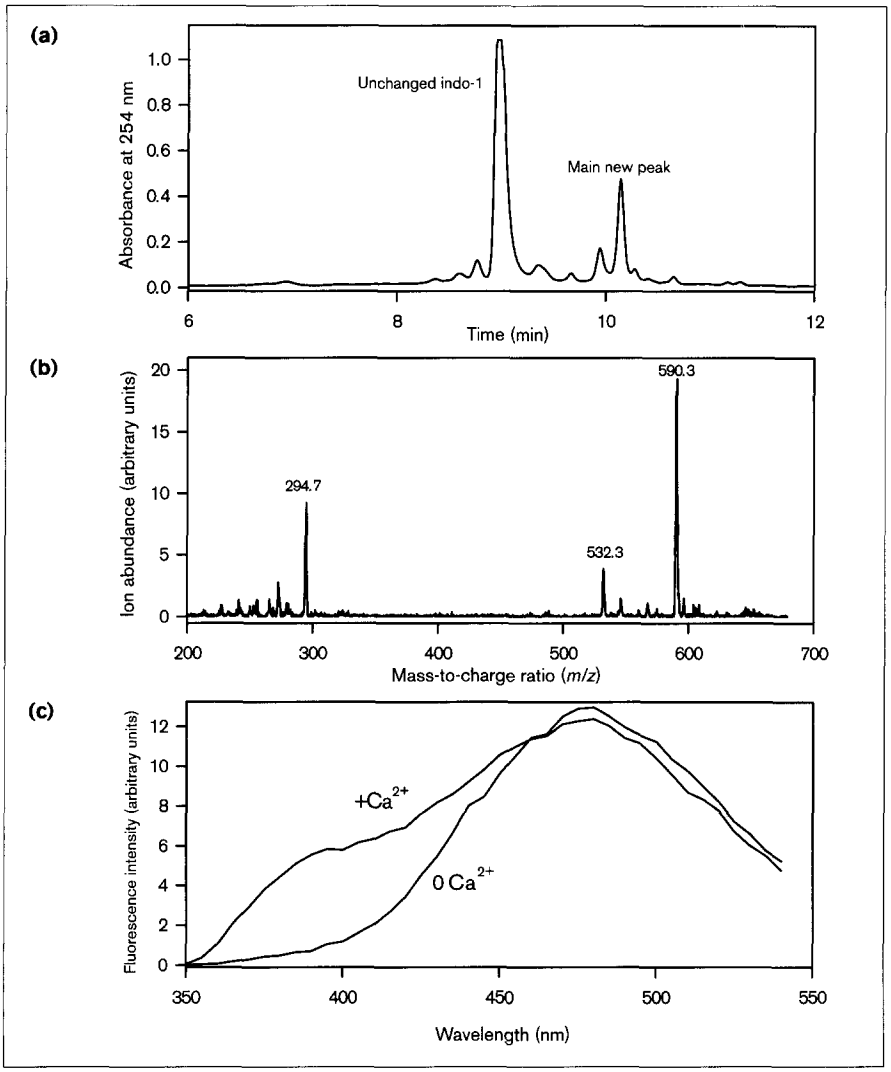
Possible photochemical reactions of indo-1 to explain photobleaching, formation of Ca^{2+}-insensitive fluorescent products, and protection by Trolox. For further information and literature references, see end of Results.

minimized further photochemical reactions of the primary products. Figure 9a is a HPLC absorbance trace after photolysis. Interesting fractions were analyzed by negative-ion electrospray mass spectrometry and fluorescence spectroscopy. Unchanged indo-1 had a mass-to-charge (*m/z*) ratio for $(M-H)^-$ of 648.4, as expected. The main new peak eluted about 1 min after indo-1. Its mass spectrum (Fig. 9b) showed *m/z* ratios of 590.3 for $(M-H)^-$ and 294.7 for $(M-2H)^{2-}$, giving a mass of 591.3 Da for the uncharged parent. This loss of 58 Da from indo-1 is consistent with replacement of $-CH_2COOH$ by –H, that is, dealkylation of the aniline nitrogen to give product G in Figure 8. A smaller mass peak at *m/z* 532.3 in Figure 9b fits with another loss of 58 Da, corresponding to some doubly-dealkylated product. The fluorescence spectra of the HPLC-isolated main product (Figure 9c) show that its emission spectrum is much less sensitive to saturating Ca^{2+} than that of indo-1. In particular, the Ca^{2+} complex has only a modest shoulder instead of a dominating peak at 400 nm. This behavior is expected from product G, because with only one rather than two carboxymethyl substituents on the anilino nitrogen conjugated to the indole, the ability of Ca^{2+} to twist the anilino N–C bond and hypsochromically shift the spectrum should be much reduced. Thus, this product indeed has the correct spectral properties to explain what happens to indo-1 upon photolysis (Fig. 5).

Discussion

Indo-1 is currently the most popular ratiometric dye for Ca^{2+} measurements with confocal microscopy, fluorescence-activated cell sorters or fast single cell photometry [1,6,7,20]. Its main advantage for these techniques is the emission shift induced by Ca^{2+} binding, which permits dual-wavelength emission ratiometric calibration using only a single wavelength of excitation. However, indo-1

Figure 9



Determination of the structure and fluorescence spectra of the Ca^{2+}-insensitive fluorescent product. **(a)** High-performance liquid chromatogram after partial photolysis under argon in 20 mM aqueous diisopropylethylammonium bicarbonate with no added Ca^{2+}. The peak eluting at 9 min is unchanged indo-1. **(b)** Negative-ion electrospray mass spectrum of the main photolysis product, eluting at 10.2 min. **(c)** Fluorescence emission spectra of the same HPLC fraction excited at 344 nm, first with 10 μM EDTA added to chelate any contaminating Ca^{2+} (0 Ca), then with 0.1 mM Ca^{2+} (+ Ca^{2+}). For further experimental details, see Materials and methods.

photobleaches faster than other dyes of the BAPTA family such as fura-2 or fluo-3. Simple photobleaching would be canceled by the ratioing process and would not cause a systematic shift in apparent $[Ca^{2+}]_i$, though it would gradually degrade the signal-to-noise ratio of the measurement. A more severe and unexpected problem with indo-1 loaded cells is the apparent decrease in baseline $[Ca^{2+}]_i$ during prolonged illumination. This phenomenon has been attributed to a cytotoxic effect [8], though cell damage is usually accompanied by increases rather than decreases of $[Ca^{2+}]_i$. Here we confirm that not only apparent basal $[Ca^{2+}]_i$ but also apparent peak $[Ca^{2+}]_i$ levels in strongly stimulated cells decrease upon continuous illumination. This result, however, is not due to toxicity, but is rather a consequence of irreversible photodegradation of the indicator. This conclusion is based on the following observations: decreases in baseline ratio values could also be observed in a cell-free preparation, when indo-1 was trapped inside liposomes or dissolved in simple buffers, and, when cells were double loaded with indo-1 and fluo-3, the peak amplitude of the fluo-3 signal was not altered after prolonged UV-laser illumination.

This tendency of indo-1 to photodegrade to a fluorescent Ca^{2+}-insensitive species was variable among cell types (W.J.J.M.S., unpublished data). If uncorrected, it can ruin the calibration of the emission ratio in terms of $[Ca^{2+}]$. These observations are somewhat analogous to those of Becker and Fay [9], who reported that fura-2 could also be converted to a relatively Ca^{2+}-insensitive fluorescent product. Becker and Fay suggested that, to minimize photodegradation, one should decrease the illumination dose and O_2 concentration. Although unnecessary illumination should certainly be reduced as much as possible, some excitation light will always be necessary to make the measurement. Anoxia is tolerable in some cell types but is

severely harmful or at least a major perturbant on most mammalian cells. Also, at least for indo-1, removal of O_2 does not prevent the dealkylation. We thus tested several antioxidants including thiols, ascorbic acid, and the vitamin E derivative Trolox [19,21,22] in an attempt to cure this problem. Whereas extracellular ascorbic acid was ineffective at the concentrations tested, Trolox greatly reduced both the overall photobleaching and the formation of the fluorescent Ca^{2+}-insensitive species in simple solution and in intact cells.

Including Trolox in the extracellular solution might thus prove a way to circumvent the problem, though care has to be taken in the use of this compound, since several studies have reported actions of Trolox on second messenger levels; a stimulation of the arachidonic acid cycle seems to be the most pronounced effect [23]. Trolox did have a slight effect on the Ca^{2+} signaling in the GH3 cells studied here, as seen by the temporary inhibition of the spontaneous oscillations upon treatment with the drug at 100 μM. However, concentrations of Trolox of 10 μM seemed adequate to prevent the alteration in ratio values, while having no major acute effects on the oscillation frequency.

In conclusion, the present study shows that care has to be taken when using indo-1 as indicator for determining cellular or subcellular [Ca^{2+}], since UV illumination will progressively deform the emission spectrum and Ca^{2+} sensitivity of the dye. The amount of deformation depends on the intensity and duration of illumination, and it occurs not only in confocal laser scanning microscopy but also during prolonged illumination from xenon or mercury arcs. Photodegradation to a fluorescent but cation-insensitive species is not a unique property of indo-1 but can also be observed in other cation indicators that contain bis(carboxymethyl)anilines, such as fura-2 [9], Ca^{2+}-Green, Mg^{2+}-Green, and analogs (S.R. Adams and R.Y.T., unpublished data). Again, Trolox seems to be significantly protective (J. Llopis, unpublished data). Still, the most satisfactory long-term solution would be to change the indicator structure to minimize the dealkylation without resorting to additives such as Trolox, whose pharmacological side effects must be tested on each new biological preparation. This is a challenge for future collaboration between mechanistic photochemists, organic chemists, and cell biologists.

Significance

UV illumination of indo-1 not only gradually photobleaches this Ca^{2+} indicator but also forms a fluorescent but relatively Ca^{2+}-insensitive compound, formed by loss of a chelating carboxymethyl side arm, which can lead to a severe underestimation of intracellular [Ca^{2+}] even with emission ratioing to cancel overall bleaching. Such photodealkylation is likely to affect most of the presently available fluorescent indicators of physiological divalent cation concentrations. Although such indicators remain the best general means for monitoring these biological signals, and we are not aware of any major biological conclusions that must be re-examined, users must be on guard against this insidious potential artifact. The ability of Trolox to protect indo-1 may stimulate testing of this and other antioxidants in a wide variety of biological systems. Ideally, the indicator structures should be modified to reduce photochemical generation of fluorescent but analyte-insensitive compounds.

Materials and methods

Cellular preparation

GH3 cells (kind gift of Dr. Hescheler, Berlin) were plated one day before the experiment on poly-L-lysine coated coverslips (0.2 μM for 45 min). The plating density was 10 000 cells per coverslip (diameter 24 mm). The culture medium consisted of HAM F-10 supplemented with 12.5 % horse serum, 2.5 % fetal calf serum (FCS), 1 % glutamine, 1 % non-essential amino acids, 0.1 % penicillin.

CLSM measurements

Before the experiment, cells were loaded in HAM F-10 medium containing 5 μM indo-1/AM, 0.02 % Pluronic F-127, 25 μM sulfinpyrazone and 3 % FCS for 30 min. at 37 °C. In experiments of double-loading with fluo-3, 5 μM fluo-3/AM was also included. After loading, cells were washed twice with HAM F-10/3 % FCS and incubated another 15 min at 37 °C to allow complete de-esterification of the internalized dye. After the post incubation, the coverslip with cells was placed in a Leiden chamber [24] and 0.5 ml Ringer's solution was added.

Cells were placed on the stage of an inverted microscope, equipped with a 40x water immersion objective (NA = 1.1) (Nikon, Tokyo, Japan), connected with a real-time UV confocal microscope (Nikon RCM-8000). All experiments were performed at room temperature. Laser excitation power at 351 nm, reaching the objective, was set at 2 μW by adjusting the laser output power and introducing neutral density filters. Fluorescence emission at 405 and 485 nm were collected simultaneously using a dichroic mirror (445 nm). 32 scans of 33 ms each were averaged per time point. Time series were acquired with a frame interval of 2 s and images at both wavelength were stored on an optical memory disc recorder (Panasonic TQ-2028F).

In some experiments bleaching properties of indo-1 were tested in a cell-free preparation, by including the free acid form of indo-1 in liposomes. Liposomes were prepared as described previously [25]. The indo-1 concentration to be trapped inside was set at 100 μM. The intra-liposomal solution contained either 0 mM Ca^{2+} and 5 mM EGTA ([ethylenebis(oxyethylenenitrilo)] tetraacetic acid) (Ca^{2+} free) or 5 mM Ca^{2+} (high Ca^{2+}).

In experiments in which a depolarizing KCl-pulse was applied, 0.5 ml of a solution containing 9 mM KCl was gently added to the bath solution, yielding a final [KCl] of 14 mM. In most experiments, half of the cell population (or liposomes) were exposed continuously during the experiments to the exciting beam, whereas the other 50 % were shielded for a given amount of time. This shielding was achieved by introducing a mask in the laser excitation pathway. This mask shielded half of the objective's field from receiving excitation light. The exact masking time is given in the figures.

Analysis of in vitro *photolysis products*

Indo-1 pentapotassium salt, 0.5 mM, was dissolved in 20 mM diisopropylethylammonium bicarbonate. A 50 μl droplet was pipetted onto the wall of a quartz cuvette, which was stoppered with a septum and

purged with argon. The droplet was irradiated at 365 nm from a UV transilluminator (Spectroline 365A) for 30 min. 10-μl aliquots were chromatographed on a 1 mm internal diameter, 15 cm long Zorbax (Rockland Technologies) octadecylsilane stationary phase, in a Michrom Bioresources narrow-bore HPLC. The solvent gradient ran linearly from 50 mM diisopropylethylamine, 100 mM acetic acid to 78 % acetonitrile in the same buffer over 10 min. Fractions detected by absorbance at 254 nm were collected and injected immediately with an infusion solution of 50 % aqueous acetonitrile into a Hewlett-Packard 5989B electrospray mass spectrometer in negative ion mode. Fluorescence spectra were recorded on a Spex Fluorolog spectrofluorometer. Emission spectra are not corrected for monochromator and photomultiplier characteristics.

Materials

Indo-1/AM and Pluronic F-127 were obtained from Molecular Probes, nonessential amino acids were from Gibco, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Aldrich, culture media and sera were from Technogenetics (Milano, Italy), and other chemicals were from Sigma.

Data analysis

Off-line analysis of both the single-wavelength signals, as well as the ratio was achieved by obtaining the signals from individual cells. All data are presented as normalized ratio values. For this, each time point was divided by the average value of the first minute baseline ratio. Subsequently, traces were analyzed by fitting a mono-exponential bleaching curve through the baseline Ca^{2+} for a population of cells. In those cells displaying spontaneous Ca^{2+} oscillations, baseline fitting was performed manually, while in the remaining cells fitting was achieved by a spreadsheet automated least square fitting [26]. All data are tested for statistical significance with a paired Student's t-test.

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