

Photoactivation and calcium sensitivity of the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2): implications for cellular NO imaging

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Abstract The fluorescent indicator of nitric oxide (NO), 4,5-diaminofluorescein (DAF-2), and its membrane-permeable derivative (DAF-2 diacetate) have been recently developed to perform real-time biological imaging of NO. In this study, we show that DAF-2 is strongly influenced by factors other than the concentration of NO itself. Using measurements with a fluorimeter as well as fluorescence microscopy, we found that the divalent cation concentration in the medium, as well as the incident light, strongly affects the ability of DAF-2 to detect NO. Calcium, in particular, enhanced the signal detection of NO released by NO donors by up to 200 times. With multiple and longer exposures to light, no bleaching of the dye was observed but, instead, a potentiation of the fluorescence response could be measured. While these two properties will affect the use and interpretation of the hitherto acquired data with this fluorescent compound, they may also open up new possibilities for its application. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Nitric oxide; 4,5-Diaminofluorescein; Fluorescence microscopy; Intracellular calcium

1. Introduction

Nitric oxide (NO) has become a species of significant biological interest due to its multiple physiological functions and general ubiquity [1–3]. NO is a small hydrophobic molecule with chemical properties that make it uniquely suitable as both an intra- and extracellular messenger.

The unstable nature of the NO molecule and its low cellular production [4,5] complicate the design of detection methods. In spite of these difficulties, several methods for detecting NO have been developed such as trapping NO with hemoglobin [6], chemiluminescence assays [7], electron paramagnetic resonance spectroscopy [8], or measurements with different electrochemical electrodes [9]. These detection methods are limited by their relatively low sensitivity and poor spatial resolution. They provide little information about the source of NO or the identification of NO-synthesizing cells. Similar to what had been established to image intracellular Ca²⁺ with fluorescent indicators, Kojima et al. [10,11] have developed NO-fluorescent indicators based on the fluorescein chromophore to allow real-time biological imaging of NO. One of these indicators, 4,5-diaminofluorescein (DAF-2), is in itself

non-fluorescent, but reacts with NO in the presence of oxygen to form the highly fluorescent triazolofluorescein (DAF-2T) which has excitation and emission maxima at respectively ~495 nm and ~515 nm [10]. A membrane-permeable form of the dye, the diacetate ester derivative (DAF-2 DA), can be taken up by the cells and hydrolyzed by cellular esterases to form again the membrane-impermeable compound DAF-2. In the present report, we commonly use the term 'DAF-2 fluorescence' to indicate the appearance of the fluorescent product of the reaction between DAF-2 and NO. This NO dye has already been employed in a series of studies using different cellular preparations (see examples in [12–22]).

In this study, we describe that the NO sensitivity of DAF-2, studied both in Ringer solutions as well as trapped intracellularly following loading with DAF-2 DA, is strongly influenced by the presence of divalent cations as well as by the incident light intensity. It thus appears that the experimental conditions for NO imaging using DAF-2 have to be rigorously controlled and that previously published results using this dye may need certain re-evaluation.

2. Materials and methods

2.1. Fluorimetric analysis of DAF-2 free in solution

The NO donors *S*-nitrosocysteine (SNC), sodium nitroprusside (SNP), and DETA NONOate (DETA/NO) were added with DAF-2 (15 μM) immediately before the beginning of each experiment to different Ringer solutions. A Ca²⁺/Mg²⁺-free Ringer solution containing (in mM) NaCl 145, EGTA 0.5, EDTA 0.5, HEPES 20, or Ringer solutions containing different concentrations of Ca²⁺ or Mg²⁺ were used. The free Ca²⁺ and Mg²⁺ concentrations were determined using the software WEBMAXC v2.10 [23]. The pH of the solutions was 7.6. The reactions were performed in Falcon Microtest[®] 96 plates (Becton Dickinson, New Jersey, USA). The volume of the reaction medium was 200 μl. Measurements were performed at 37°C with a fluorimeter (PerSeptive Biosystems, Cytofluor Multi Well Plate reader 4000) with excitation wavelength at 485 nm (20 nm bandwidth) and emission wavelength at 530 nm (25 nm bandwidth). Continuous data acquisition was performed for 60 min on a PC Pentium Pro (333 MHz). The data are presented as mean values ± S.E.M. Differences between means were analyzed using Student's *t*-test for paired experiments.

2.2. Determination of nitrite concentration

The influence of divalent cations on the release of NO by different NO donors (SNC, SNP, DETA/NO) was verified by measuring the nitrite concentration (NO₂⁻) on a spectrophotometer (540 nm) by the Griess reaction [24].

2.3. Intracellular DAF-2 imaging

Human embryonic kidney (HEK293) cells were grown at 37°C on polylysine-coated coverslips in minimal essential medium supplemented with 10% horse serum and 1% gentamicin. The cells were loaded during 15–20 min at 37°C by adding 10 μM DAF-2 DA in

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the culture medium. Once loaded with DAF-2 DA, the cells were rinsed with the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Ringer solution and placed in a perfusion chamber designed for rapid exchange of solutions [25]. The cells were continuously perfused at room temperature. DAF-2 fluorescence was excited using a 490 nm (20 nm bandwidth) filter (Omega Optical, Brattleboro, VT, USA) and observed through a 515 nm long-pass emission filter.

Measurements were performed on the stage of an inverted epifluorescence microscope (Nikon, Tokyo, Japan) using a 40×1.3 N.A. oil immersion fluor objective lens (Nikon, Tokyo, Japan). The excitation light was selected using a fast filter wheel (Sutter Instruments, Novato, CA, USA) and the fluorescence was detected using a Gen III+ intensified CCD camera (VideoScope Int., Washington, DC, USA). Acquisition and digitization of video images, as well as time series were computer-controlled using the software Metafluor (Universal Imaging, West Chester, PA, USA) running on a PC Pentium computer (333 MHz). For each measurement, four video frames were averaged. Up to 20 individual cells were simultaneously analyzed in the selected field of view.

Two modes of illumination were used: a low and a high light level mode. (1) Low light level mode: the 490 nm light output from the 100 W xenon lamp (Nikon) was reduced to 0.03% prior to reaching the specimen plane by inserting neutral density filters (Omega Optical) in the light path. The resulting illumination power at 490 nm measured at the back focal plane of the $40\times$ objective lens using a commercial optical power meter (model 350, VDT, Hawthorne, CA, USA) was 5–10 μW . Brief exposures (~ 200 ms) at 5–10 s time intervals were used. (2) High light level (photoactivation) mode: the light flux at 490 nm light was set at 3 or 10% (i.e. 100–300-fold higher light flux than in the low light level mode) and exposure times of 1–25 s were used. Images were not acquired during these exposures.

2.4. Intracellular Fura-2 imaging

HEK293 cells were loaded with 6 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) for 25 min at 37°C in the culture medium. Fluorescence excitation ratios proportional to Ca^{2+} concentration changes were obtained by sequential illumination at 340 and 380 nm with the excitation intensity of the xenon lamp attenuated to 0.03% of the initial intensity (low light level mode). A two-frame average was performed for each acquired image.

2.5. Chemicals

The stock solutions of DAF-2 and DAF-2 DA, 5 mM (Calbiochem, Foster City, CA, USA); 2,7-dichlorofluorescein, 1 mM (Molecular Probes, Eugene, OR, USA); Fura-2 AM, 2 mM (Molecular Probes, Eugene, OR, USA); ionomycin, 4 mM (Sigma-Aldrich, Steinheim, Germany) were dissolved in dimethyl sulfoxide. The stock solutions of NO donors were prepared as follows: SNC (1 mM) was prepared on ice from the combination of equimolar amounts of L-cysteine hydrochloride and sodium nitrite [26]; SNP (20 mM) (Sigma-Aldrich, Steinheim, Germany) was prepared in H_2O ; DETA/NO (6 mM) (Alexis Biochemicals, San Diego, CA, USA) was prepared directly in the selected Ringer solution. The NO donors were then further diluted to their final concentrations in the respective Ringer solutions used for particular experiments (for compositions see above).

3. Results

3.1. NO detection using DAF-2 is sensitive to divalent cations

We first examined the effects of Ca^{2+} on DAF-2 fluorescence in experimental conditions mimicking NO monitoring in extracellular environments such as interstitial or biological fluids [16,27]. These environments would typically contain

millimolar concentrations of Ca^{2+} . With the help of a fluorimeter, we monitored the increase of fluorescence levels over a period of 60 min following the addition of 15 μM DAF-2 to a Ringer solution containing respectively 0.5 mM SNC, 1.5 mM DETA/NO or 10 mM SNP, three different NO

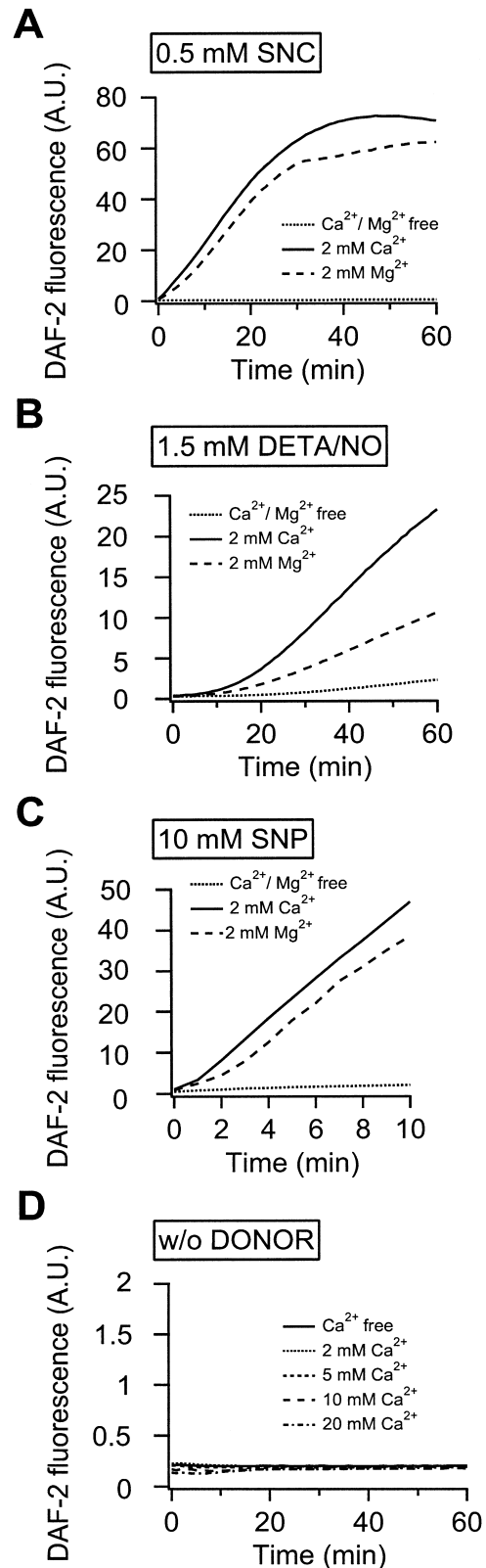


Fig. 1. Effect of divalent cations on the fluorescence response of DAF-2. Representative experiments where DAF-2 (15 μM) fluorescence was monitored during 60 min in the presence of NO generated by (A) SNC (0.5 mM), (B) DETA/NO (1.5 mM), (C) SNP (10 mM) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solutions or in solutions containing either 2 mM Ca^{2+} or 2 mM Mg^{2+} . D: Increasing concentrations of Ca^{2+} (2–20 mM) were tested on DAF-2 (15 μM) in the absence of NO donors in the Ringer solution.

donors of various chemical structure and reactivity (Fig. 1A–C). Different time courses of DAF-2 fluorescence increase were recorded in a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Ringer solution versus in solutions containing 2 mM Ca^{2+} or Mg^{2+} . Fig. 1 shows that the ionic composition of the Ringer solution strongly influenced the fluorescence level of DAF-2: DAF-2 fluorescence intensity was 170 ± 3-fold increased in the presence of 2 mM Ca^{2+} with NO generated by 0.5 mM SNC, 10 ± 1-fold for 1.5 mM DETA/NO, and 22 ± 2-fold with 10 mM SNP compared to a divalent cation-free solution ($n = 5$ for each donor and experimental condition). The increase in DAF-2 fluorescence in the presence of Ca^{2+} was not caused by an increase in NO production by the donors themselves. NO production measured with the Griess reaction [24] showed that 0.1 mM SNC produced 7.75 ± 0.07 versus 7.55 ± 0.45 μM nitrites in the presence or the absence of 2 mM Ca^{2+} respectively ($n = 5$,

NS), whereas 10 mM SNP produced 17.60 ± 0.51 versus 16.73 ± 1.11 μM nitrites ($n = 5$, NS) and 1.5 mM DETA/NO produced 48.54 ± 3.75 versus 49.98 ± 2.39 μM nitrites ($n = 5$, NS) in the presence or the absence of 2 mM Ca^{2+} respectively.

An increase in DAF-2 fluorescence was also observed in the presence of Mg^{2+} in the Ringer solution (Fig. 1A–C). Indeed, when 2 mM Mg^{2+} was present in the 200 μl reaction Ringer solution, DAF-2 fluorescence intensity was increased by a factor of 150 ± 2 -fold, 5 ± 1 -fold and 19 ± 3 -fold in the presence of NO generated respectively by 0.5 mM SNC, 1.5 mM DETA/NO or 10 mM SNP (Fig. 1A–C). As for Ca^{2+} , Mg^{2+} did not alter the ability of the donors to release NO (data not shown).

Fig. 1D shows that, in the absence of NO donors, Ca^{2+} at concentrations up to 20 mM had no effect on DAF-2 fluorescence intensity (0.215 ± 0.005 versus 0.204 ± 0.008 with 20 mM Ca^{2+} ($n = 5$, NS)). Thus the effect of Ca^{2+} and Mg^{2+} does not seem to be on the background DAF-2 fluorescence but on its sensitivity to NO, the presence of NO being required to observe an increase in fluorescence intensity. Therefore the presence of Ca^{2+} or Mg^{2+} in the reaction medium appears to enhance the conversion of DAF-2 into its fluorescent product (DAF-2T) in the presence of NO regardless of the type of NO donor.

Finally, we tried to quantify the changes in the detection threshold of NO released by the different NO donors by the presence of Ca^{2+} in the reaction medium. DAF-2 fluorescence intensity values were taken 30 min after the beginning of the reaction (Fig. 2). In the absence of Ca^{2+} , 100 μM SNC was necessary to detect NO production whereas in the presence of 2 mM Ca^{2+} , 1 μM was sufficient to observe a significant increase of NO produced over time using 15 μM DAF-2 (Fig. 2A). In the case of SNP and DETA/NO, the detection threshold was respectively 5- and 20-fold more sensitive in the presence of Ca^{2+} (Fig. 2B,C).

3.2. Effect of light on NO detection using DAF-2

In the presence of 0.1 mM SNC and 15 μM DAF-2, we could observe an unexpected and major increase in the fluorescence intensity (Fig. 3A) when the number of readings per minute of the fluorimeter was increased from one (0.400 ± 0.005) to three (0.590 ± 0.009) or five reads (1.220 ± 0.005) ($n = 4$). Each read had a duration of 0.2 s. The same effect was observed in the presence of NO released by SNP or DETA/NO (data not shown). This phenomenon was never observed in the absence of NO donors in the Ringer solution (Fig. 3A). These measurements showed that a NO-dependent enhancement of DAF-2 fluorescence proportional with the duration of light exposure occurs in a cell-free environment.

In order to verify if these observations could be observed intracellularly, DAF-2 was loaded into HEK293 cells using its membrane-permeable diacetate derivative DAF-2 DA [10] and visualized by fluorescence microscopy. As originally described [10], DAF-2 fluorescence associated with cells was found to be extremely weak in the absence of NO. Initial experiments showed that when observed in low light level conditions typically used e.g. for intracellular Ca^{2+} imaging (see Section 2), superfusion of the cells with different NO donors as for example SNC (0.1 mM) consistently failed to elicit any sizable change in fluorescence (data not shown). No bleaching of the dye was observed under these low light level conditions.

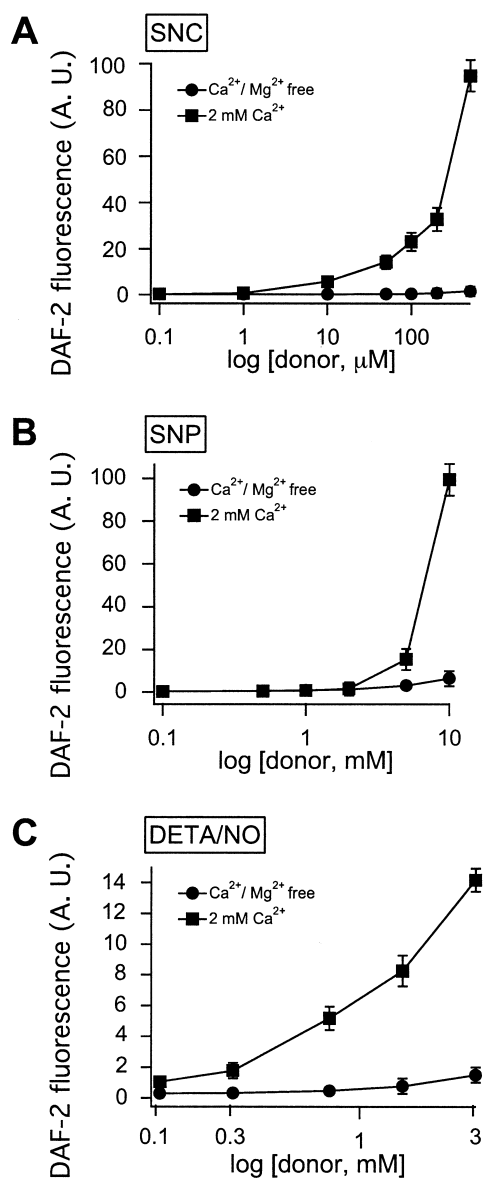
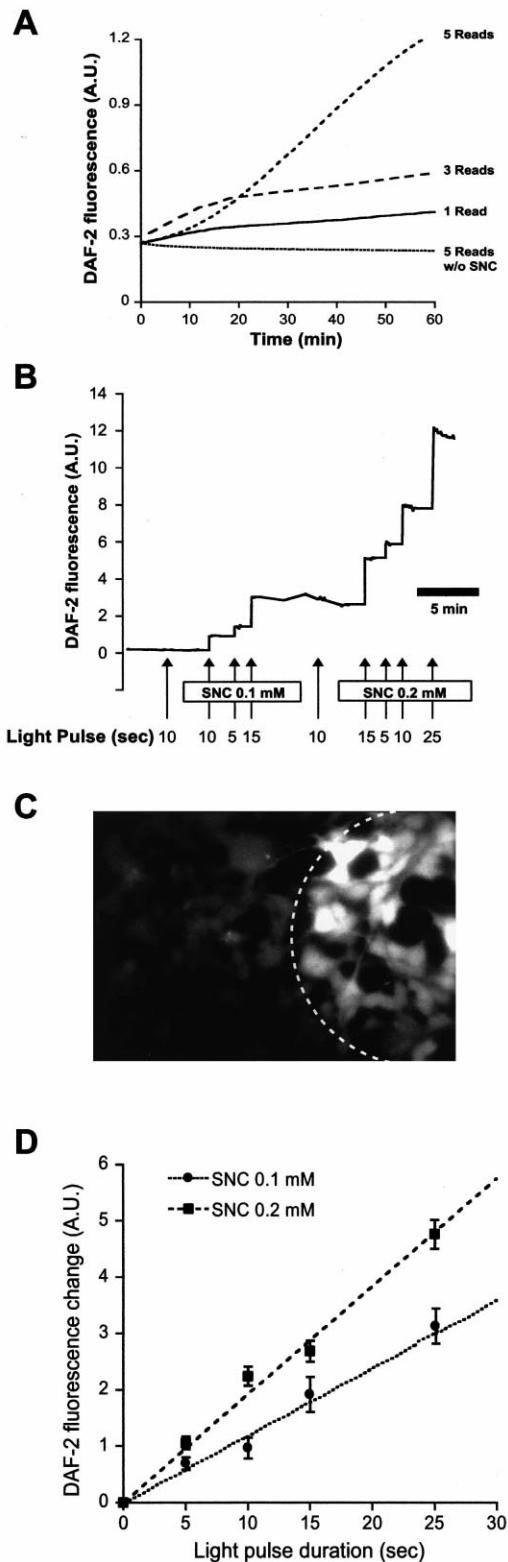


Fig. 2. Calcium sensitivity of DAF-2 fluorescence response. Dose-response curves of three NO donors (SNC, SNP, DETA/NO) in the presence or not of 2 mM Ca^{2+} in the Ringer solution at 30 min ($n = 5$ for each concentration of NO donor and experimental condition).



When brief pulses of intense light at 490 nm (i.e. high light level mode, with 100–300-fold higher light intensity) were applied, they were found to reproduce what we had seen with the fluorimeter (Fig. 3A,B). In the presence of SNC, images taken at low light level immediately after the high light pulse showed an enhanced fluorescence, instead of the expected weaker signal due to photobleaching. Note that no images

Fig. 3. Photosensitivity of DAF-2 fluorescence response. A: Representative recordings of DAF-2 fluorescence made with increasing duration of illumination on the fluorimeter (1, 3 and 5 reads/min of 0.2 s each) as indicated in the graph, in the presence of 0.1 mM SNC. In comparison, a curve is presented for the longer light exposure time (5 reads, 1 s) but in the absence of SNC (DAF-2 alone). B: DAF-2 DA loaded in HEK293 cells observed by fluorescence microscopy. Pulses of high intensity light with 5, 10, 15, or 25 s duration were applied in the presence or in the absence of SNC. The time of application and the concentration of SNC are indicated in the graph. C: Image of HEK293 cells loaded with DAF-2 DA and perfused with 0.1 mM SNC. At the end of the experiment shown in B, the field of view was shifted to the right uncovering cells that were out of the field of illumination and had therefore not been subjected to the high light pulses, but that had nevertheless been superfused with SNC. The dashed line in overlay indicates the border of the field of view used during the experiment. D: Relationship between the high light pulse duration (s) and the change in DAF-2 fluorescence induced by 0.1 mM and 0.2 mM SNC. Linear regression analysis yielded correlation coefficients of 0.974 and 0.994 for 0.1 mM SNC and 0.2 mM SNC, respectively. Data are taken from five separate experiments.

were recorded *during* the high light pulses because the high intensity would cause the camera to saturate.

Once again, the high light pulses per se did not affect DAF-2 in the absence of the NO donor (Fig. 3A,B). As seen in Fig. 3B,D, the increase in fluorescence was dependent both on the duration of the high light pulse and on the presence and concentration of the NO donor (SNC). An example of the dramatic effect of illumination on DAF-2 is shown in Fig. 3C where the field of view used during the experiment shown in Fig. 3B was moved sideways to uncover cells that had remained protected from the light during the preceding manipulations, but that had nevertheless experienced the same SNC perfusion.

As a control, 2,7-dichlorofluorescein loaded into cells using its membrane-permeant diacetate form and used to detect reactive oxygen species [28] was tested with the same paradigm as for DAF-2 (low versus high light mode). 2,7-Dichlorofluorescein showed no photoactivation in the presence of NO (data not shown), indicating that the observation described above with DAF-2 again specifically applies to the interaction of NO with DAF-2.

3.3. Effect of intracellular Ca^{2+} concentration on NO detection using DAF-2

We finally determined whether the strong effect of Ca^{2+} on DAF-2 fluorescence described above for the extracellular environment was also relevant for intracellular NO measurements. We first performed fluorimetric measurements in conditions where Mg^{2+} was set at 1 mM and Ca^{2+} varied over the range 50 nM–1 mM, which apply to the cytosolic, mitochondrial, and the endoplasmic reticulum environments [29–31]. Under these conditions, Ca^{2+} also markedly enhanced the NO-evoked DAF-2 fluorescence response (Fig. 4A). For example, the change in Ca^{2+} from 50 nM to 1 μ M enhanced DAF-2 fluorescence by a factor of 4.45-fold in the presence of NO released by 0.5 mM SNC. To verify if this could also be observed in living cells, HEK293 cells loaded with DAF-2 DA were treated with SNC (0.1 mM) and high light pulses were applied (Fig. 4B). The cells were then permeabilized using ionomycin (4–6 μ M), a treatment that abruptly increases the cytosolic Ca^{2+} concentration as measured using the intracel-

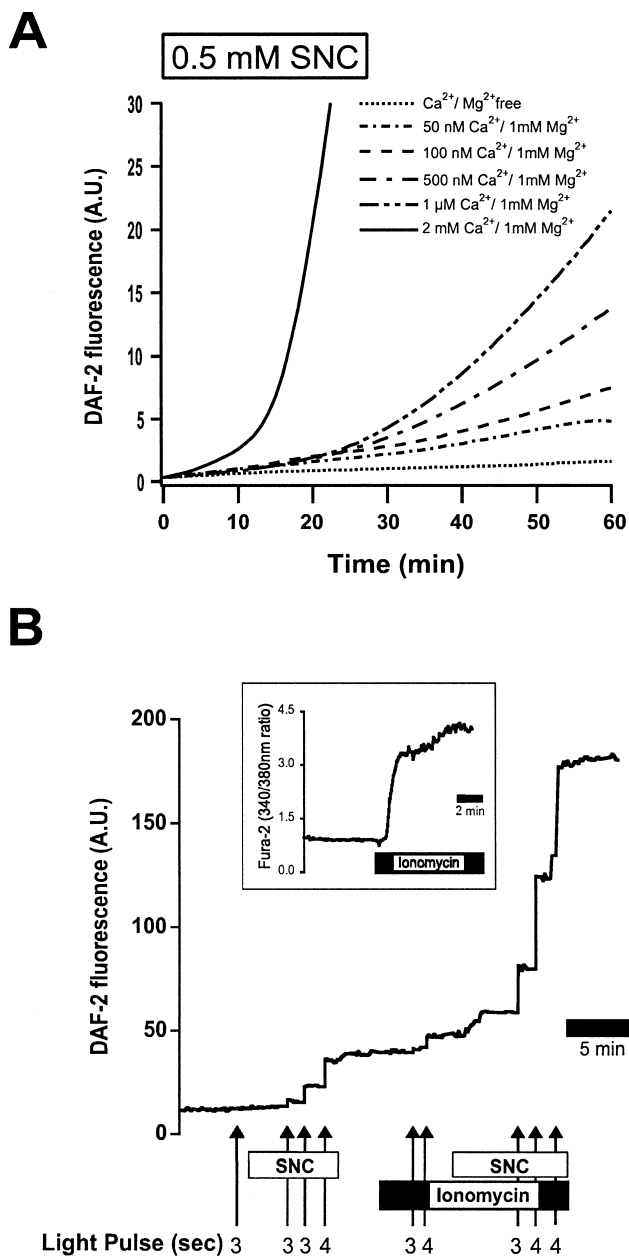


Fig. 4. Enhancement of DAF-2 fluorescence by physiological calcium concentrations. A: Representative experiment where DAF-2 (15 μM) fluorescence was monitored during 60 min in the presence of NO generated by SNC (0.5 mM) in Ca²⁺/Mg²⁺-free solution or in solutions containing different Ca²⁺ concentrations (from 50 nM to 2 mM) in the presence of 1 mM Mg²⁺. B: HEK293 cells loaded with DAF-2 DA were observed by fluorescence microscopy and perfused with a Ringer solution containing 2 mM Ca²⁺. Pulses of high intensity light of 3 or 4 s duration were applied in the presence or absence of SNC (0.1 mM) as indicated in the graph. Ionomycin (6 μM) was perfused to permeabilize the cell membrane and increase the cytosolic Ca²⁺ concentration. Pulses of light were repeated first in the absence and then in the presence of SNC (0.1 mM) and showed an enhanced response of the dye during ionomycin treatment. The plot represents a typical experiment out of three. Inset: Effectiveness of ionomycin treatment on cytosolic Ca²⁺ as measured using the intracellularly loaded Ca²⁺-sensitive probe Fura-2. Data are presented as fluorescence excitation ratio changes, proportional to Ca²⁺ concentration.

lularly loaded Ca²⁺-sensitive probe Fura-2 (Fig. 4B, inset). The ionomycin treatment had no effect on DAF-2 fluorescence in the absence of SNC or in low light level observation conditions, showing once again that the fluorescence of DAF-2, or the product of its reaction with NO, was not affected by Ca²⁺. However, the appearance of the fluorescent product evoked by high light pulses in the presence of SNC was enhanced by a factor of 3.45 ± 0.28 (18 cells from three different experiments) in ionomycin-permeabilized cells with elevated cytosolic Ca²⁺, consistent with the results obtained from fluorimetric analyses. The presence of NO was again mandatory to observe this phenomenon.

4. Discussion

Few methods exist that allow the detection of NO production with cellular resolution, therefore the development of the fluorescent indicator DAF-2 [11] capable of sensing the ambient NO concentration was considered a major advance. The present study shows that DAF-2, although capable of reacting with NO produced by a variety of donors *in vitro*, suffers from at least two flaws that make it problematic to use it as a fluorescent indicator for intracellular and extracellular NO measurements.

First, DAF-2 fluorescence intensity was increased in the presence of divalent cations. Indeed, in the presence of NO, Ca²⁺ and Mg²⁺ were found to increase DAF-2 fluorescence in Ringer solution by a factor of 200. The enhancing effect of Ca²⁺ on DAF-2 fluorescence was also observed in living cells and amounted to ~3.5-fold when the cell membrane was permeabilized using the Ca²⁺ ionophore ionomycin. However, Ca²⁺ had no effect on DAF-2 fluorescence in the absence of NO, both in fluorimetric measurements and in living cells loaded using DAF-2 DA. This observation probably indicates that divalent cations interact with the dye–NO complex and favors the reaction toward the fluorescent triazolofluorescein product.

The consequences of the Ca²⁺ sensitivity of DAF-2 are multiple. In living cells, resting state cytosolic free Ca²⁺ is maintained at a very low level – typically in the 100 nM range – and can abruptly increase by a factor of > 10 upon agonist or electrical stimulation. Diacetate or acetoxyethyl esters of dyes are well known to accumulate in subcellular compartments [31] such as mitochondria where free Ca²⁺ is typically found at a few micromolar [29], or the endoplasmic reticulum where free Ca²⁺ approaches the millimolar [30,31].

Moreover, because of the high diffusivity of NO, the site of NO production within the cell could well differ from the site of its detection by DAF-2, which will occur preferentially in a Ca²⁺-rich environment, such as mitochondria. Because many physiological signals leading to NO production occur by an elevation of cytosolic Ca²⁺ which in turn activates NOS I or NOS II [32], the Ca²⁺ sensitivity of DAF-2 renders it difficult to distinguish a Ca²⁺ increase from an increase in NO production.

The second unexpected property of DAF-2 is its photosensitivity. From the observations made with cells loaded with DAF-2 DA, a certain level of luminous flux reaching the dye in the presence of NO was found to be mandatory to yield detectable fluorescent products. In the dark, NO did not appear to react with DAF-2. This observation was made both with DAF-2 loaded into living cells and with a fluorimeter.

Therefore, the very light used to excite the fluorophore for observation seems to interact with DAF-2 molecules to produce more fluorescent product. The first consequence is that quantification of NO production is extremely delicate and prone to errors. Working under true low light level conditions does not allow the detection of NO production by DAF-2. The light intensity used to photoactivate DAF-2 in the present study (500–3000 μW) is routinely used for visualization in laser scan confocal microscopy [33]. Moreover, the relatively intense light flux necessary for DAF-2 photoactivation is potentially harmful for living cells, and could lead to free radical formation [34].

The interpretation of the first studies published so far that used DAF-2 with cells (for example see [12–22]), which involved Ca^{2+} changes, confocal microscopy, or subcellular NO detection, should probably be re-examined in view of the peculiar properties of DAF-2 described in the present study. Nevertheless, the photochemistry of DAF-2 is certainly interesting and deserves further investigation. Indeed, the photochemical properties of this NO-sensitive dye make it potentially useful in photodynamic therapy to target NO-producing tumor cells. The mechanisms underlying the reaction of NO with DAF-2 are complex [10], but it appears conceivable that the transition to a triplet state of the DAF-2 molecule, necessary for its interaction with NO^\bullet (or NO_2^\bullet) radical, is favored by light.

In conclusion, we suggest that DAF-2, developed for fluorescent NO monitoring, will be difficult to use for fluorescent microscopic studies with living cells. In order to obtain reliable information about NO production in cells, the use of this dye would require careful adjustment of the experimental conditions and complete control of the intracellular Ca^{2+} changes, which is only rarely possible.

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