Orthogonality of Calcium Concentration and Ability of 4,5-Diaminofluorescein to Detect NO*

Noriyuki Suzuki, Hirotsu Kojima, Yasuteru Urano, Kazuya Kikuchi, Yasunobu Hirata, and Tetsuo Nagano†‡

From the †Graduate School of Pharmaceutical Sciences and ‡The Second Department of Internal Medicine, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received for publication, August 24, 2001, and in revised form, October 10, 2001
Published, JBC Papers in Press, October 18, 2001, DOI 10.1074/jbc.M108195200

We have developed diaminofluoresceins (DAFs) and diaminorhodamines as fluorescent indicators for NO based on the specific reaction of the aromatic vicinal diamines with NO. Among them, 4,5-diaminofluorescein (DAF-2) is widely used for real-time biological imaging of NO in cultured cells or tissues by many researchers. Contrary to a recent report of divalent cation sensitivity and photoactivation of DAF-2 (Brouillet, M. C., Randin, O., and Chatton, J. Y. (2001) FEBS Lett. 491, 227-232), our study using NO gas itself reveals that the reaction of DAF-2 and NO is completely independent of Ca²⁺ and Mg²⁺ at physiological concentrations. Ca²⁺ enhances not the conversion of DAF-2 into its fluorescent product (DAF-2 triazole) but the release of NO from NO donors. Therefore it is concluded that DAF-2 can provide reliable information on NO production in biological systems regardless of the dynamic changes of Ca²⁺ concentration.

 Nitric oxide is a gaseous free radical that plays a role as an intracellular second messenger and a diffusable intercellular messenger (1). Because of its instability and low concentration in biological systems, it had been difficult to perform direct NO detection with satisfactory sensitivity. We have developed diaminofluoresceins and diaminorhodamines as fluorescent indicators for NO that overcome those difficulties and allow bioimaging of NO with high spatial and temporal resolution (2-6). 4,5-Diaminofluorescein (DAF-2) has particularly advantageous properties for fluorescence microscopy, and DAF-2 and its derivatives have been widely used in practical studies using cultured cells or tissues (7-18).

However, Brouillet et al. (19) recently reported divalent cation sensitivity and photoactivation of DAF-2. They carried out fluorometric analysis and intracellular NO imaging by use of DAF-2 with NO donors such as S-nitrosocysteine (SNC), DETA/NO, and sodium nitroprusside (SNP). They suggested that DAF-2 fluorescence is influenced by factors other than the concentration of NO itself. In their experiments using several NO donors, apparent potentiation of the fluorescent response of DAF-2 in the presence of Ca²⁺, Mg²⁺, or incident light was observed. They proposed that divalent cations interact with the DAF-2-NO complex and favor the reaction toward the fluorescent product and that the light used to excite the fluorophore interacts with DAF-2 molecules to produce more fluorescent product. However, they did not consider the possibility that the divalent cations and intense light they used interacted with the NO donors, not with DAF-2. It is generally accepted that the NO release rates of many NO donors (especially S-nitrosothiols) are strongly influenced by divalent cation concentration in the medium (20). Furthermore, S-nitrosothiols absorb visible light in the range of λ = 550-600 nm (although the molar absorption coefficient is low), and it is known that irradiation of S-nitrosothiol with light (λ = 550 nm) results in the generation of thyl radical and NO (21).

In this report, we carried out fluorometric analysis by use of DAF-2 with NO gas to examine the influence of divalent cations on the reaction of DAF-2 and NO itself. In this simple reaction system, we established that the ability of DAF-2 to detect NO is independent of Ca²⁺ and Mg²⁺ at physiological concentrations.

EXPERIMENTAL PROCEDURES

Fluorometric Analysis with DAF-2—Fluorescence measurements were performed with a PerkinElmer LS-50B Luminescence Spectrometer with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The slit width was 2.5 nm for both excitation and emission. NO solution or NO donors SNC, DETA/NO, and SNP were added to Ringer’s solution containing DAF-2 (15 μM) at the beginning of each experiment. A Ca²⁺/Mg²⁺-free Ringer’s solution containing 145 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, and 20 mM HEPES or Ringer’s solution containing 2 mM Ca²⁺ or Mg²⁺ was used. The free Ca²⁺ and Mg²⁺ concentrations were determined using the software WEBMAXC v2.10 (22). Reactions were performed in a 10-mm quartz cuvette at 37 °C at pH 7.6. The volume of the reaction medium was 3 ml. All reactions were carried out at least in duplicate, and their exact reproducibilities were confirmed. The data shown in the figures are representative of these experiments.

UV Absorption Analysis of SNC Conversion to Cystine and NO Generation from SNC—UV absorption analyses of the conversion of SNC to cystine were performed with a Shimadzu UV-1600 UV-visible spectrophotometer. SNC was added at 0.5 mM to the different Ringer’s solutions at the beginning of each experiment, and the absorbance was monitored at λ = 336 nm. Reactions were performed in a 10-mm quartz cuvette at 37 °C at pH 7.6. The volume of the reaction medium was 3 ml. All reactions were carried out at least in duplicate, and their exact reproducibilities were confirmed. The data shown in the figures are representative of these experiments.

Chemicals—NO solution was prepared by bubbling NO through HEPES buffer for 2 min after the buffer had been deoxygenized by bubbling argon through it for 1 h, and NO concentration of the NO solution

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to

† To whom correspondence should be addressed. Tel.: 81-3-5841-4856; Fax: 81-3-5841-4855; E-mail: tsn@mol.fu.u-tokyo.ac.jp

‡ The abbreviations used are: DAF-2, 4,5-diaminofluorescein; SNC, S-nitrosocysteine; DETA/NO, diethylenetriamine NONOate; SNP, sodium nitroprusside; DCFH, dichlorofluorescin.

Published, JBC Papers in Press, October 18, 2001, DOI 10.1074/jbc.M108195200

This paper is available online at http://www.jbc.org
was determined by the Griess method. SNC was prepared according to the method in the literature (23) and dissolved in Ringer’s solution. DETA/NO (Dojindo Laboratories, Kumamoto, Japan) was dissolved in 0.1 M NaOH aqueous solution. DAF-2 was dissolved in Me2SO to obtain a 10 mM stock solution. The NO donors and DAF-2 were then further diluted to the required concentration in the respective Ringer’s solution for particular experiments.

RESULTS

We first examined the influence of Ca2+ and Mg2+ on the reaction of DAF-2 and NO. To rule out involvement of factors other than DAF-2 and NO, we used NO gas itself instead of the NO donors that Broillet et al. (19) used. Time courses of DAF-2 fluorescence increase by various concentrations of NO were recorded in Ca2+/Mg2+/-free Ringer’s solution and in Ringer’s solution containing 2 mM Ca2+ or Mg2+. Almost identical time courses of DAF-2 fluorescence increase were obtained for each NO concentration regardless of whether divalent cations were present or not as shown in Fig. 1. These results clearly show that the increase of DAF-2 fluorescence intensity is independent of the presence of Ca2+ or Mg2+.

Next we repeated the experiments using NO donor such as SNC, DETA/NO, or SNP instead of NO gas, essentially repeating the work of Broillet et al. (19) did in their report. The time courses of fluorescence clearly varied depending on the presence of divalent cation in the case of SNC as the NO donor as shown in Fig. 2. This is in agreement with the finding of Broillet et al. (19); while in our experiments, time courses were not significantly influenced by divalent cations in the case where DETA/NO and SNP were used as NO donors (not shown). Broillet et al. (19) hypothesized in their report that this enhancement of fluorescence intensity is due to the interaction of the divalent cations with the DAF-2/NO complex and favors the reaction toward the fluorescent product DAF-2 triazole. However, this proposal is in conflict with our results by the use of NO gas mentioned above.

To explain the difference between the results by the use of NO gas and the results by the use of chemical compounds that generate NO gas contrary to Broillet et al. (19), we hypothesized that the divalent cations enhance not the conversion of DAF-2 into its fluorescent product (DAF-2 triazole) but the release of NO from NO donors. This idea is supported by a previous report that the NO release rate from SNC is strongly influenced by certain divalent cations attributable to coordination of the cation to SNC (20). We therefore monitored the conversion of SNC to cystine in the presence and absence of divalent cations by measuring the absorption of SNC at 436 nm. As shown in Fig. 3a, the conversion of SNC to cystine was greatly enhanced by Ca2+. The magnitude of the DAF-2 fluorescence enhancement by Ca2+ and that of the SNC decay enhancement by Ca2+ were very similar and consistent with each other. Next we directly monitored the NO generation that occurred coincidentally with the conversion of SNC to cystine by quantitative determination of NO2 using the Griess method. We used the TCI NOX-1000 system because rapid determination was required for this experiment. As shown in Fig. 3b, we also confirmed that the NO generation from SNC was greatly enhanced by Ca2+ and that the generation of NO in the presence or absence of divalent cation was consistent with the SNC decay in the respective condition monitored by measuring the absorption of SNC. These results demonstrate that the divalent cation enhances NO generation from SNC and consequently enhances the increase of DAF-2 fluorescence intensity. In the case where DETA/NO and SNP were used as NO donors, conversion of NO donor and NO generation was not significantly influenced by divalent cations compared with the case of SNC as the NO donor (not shown). These results of UV absorption analysis are also consistent with those of the corresponding fluorometric analysis.

DISCUSSION

Our experiments presented in this paper by the use of NO gas itself instead of the NO donors clearly showed that the increase of DAF-2 fluorescence intensity is independent of the presence of Ca2+ or Mg2+. This result means that the rate constants of each reaction step in the reaction of DAF-2 and NO under aerobic condition are not influenced by the presence of the divalent cations. Broillet et al. (19) reported that the increase of DAF-2 fluorescence intensity was enhanced 170-fold with NO generated by SNC in the presence of Ca2+ compared with the absence of Ca2+. They also reported that the enhancement was smaller with DETA/NO (10-fold) and with SNP (22-fold) in the presence of Ca2+ compared with the absence of Ca2+ (19), although the extent was much different from our data. If Ca2+ actually enhances the conversion of DAF-2 into its fluorescent product, the magnitude of the reaction enhancement by Ca2+ should be similar for each NO donor. Therefore these various magnitudes of the DAF-2 fluorescence enhancement by Ca2+ themselves indicate that their interpretation is not correct. From these various magnitudes of the DAF-2 fluorescence enhancement by Ca2+ with the respective NO donors, we hypothesized that the Ca2+ interacts with the respective NO donors, not the DAF-2, its NO adduct, or NO itself. This hypothesis was confirmed by the following UV absorption
**Fig. 3.** a, decay of SNC (0.5 mM) in the absence of divalent cation (→) and in the presence of 2 mM Ca\(^{2+}\) (←) or Mg\(^{2+}\) (−). Absorbance of SNC (λ = 336 nm) was monitored during 60 min. b, NO generation from SNC (0.5 mM) corresponding to NO\(_2\) concentrations of SNC solution in the absence of divalent cation (○) and in the presence of 2 mM Ca\(^{2+}\) (●) or Mg\(^{2+}\) (■) were determined by the Griess method.

**Scheme 1.** Reaction scheme of DAF-2 with NO in the presence of Ca\(^{2+}\).

analysis. The conversion rate of the NO donors monitored by UV absorption and the NO release rate of NO donors monitored by the Griess method were greatly influenced by the divalent cations; that is, the amount of NO itself generated by the NO donors is varied in the presence of divalent cations.

From these results, we concluded that the enhancement of DAF-2 fluorescence in the presence of divalent metal cations reported by Broillet et al. (19) was derived from the interaction of the metal cation with the NO donor, not the DAF-2, its NO adduct, or NO itself as illustrated in Scheme 1.

Broillet et al. (19) also reported the enhancement of DAF-2 fluorescence by intense light. However, it is well known that irradiation of S-nitrosothiols with light (λ = 550 nm) results in the generation of the thyl radical and NO (21), so it is likely that irradiation of DAF-2 with SNC would result in an increase of NO release from SNC, leading to the increase of DAF-2 fluorescence intensity. Broillet et al. (19) used 2,7-dichlorofluorescein (DCFH) as a control in their photoactivation experiments, but the sensitivities for NO of DAF-2 and DCFH are significantly different. Therefore, we consider that DAF-2, which is easy to use for fluorescence microscopic studies with living cells without complex techniques, can provide reliable information on NO production regardless of the dynamic changes of Ca\(^{2+}\) concentration in biological systems. However, experiments using NO donors, especially SNC, will need to take account of changes in divalent cations irrespective of the indicator being used.

**Acknowledgment**—We thank M. Morita for helpful advice about NO determination using the Griess method.

**REFERENCES**

1. Packer, L. (ed) (1996) *Methods in Enzymology*, Vol. 269, Academic Press, Inc., San Diego, CA
2. Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., and Nagano, T. (1998) *Anal. Chem.* 70, 2446–2453
3. Kojima, H., Sakuragi, K., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., and Nagano, T. (1998) *Chem. Pharm. Bull. (Tokyo)* 46, 373–375
4. Kojima, H., Urano, Y., Kikuchi, K., Higuchi, T., Hirata, Y., and Nagano, T. (1999) *Angew. Chem. Int. Ed. Engl.* 38, 3209–3212
5. Kojima, H., Hirtani, M., Urano, Y., Kikuchi, K., Higuchi, T., and Nagano, T. (2000) *Tetrahedron Lett.* 41, 69–72
6. Kojima, H., Hirtani, M., Nakatsubo, N., Kikuchi, K., Urano, Y., Higuchi, T., Hirata, Y., and Nagano, T. (2001) *Anal. Chem.* 73, 1967–1973
7. Kojima, H., Hirata, M., Kudo, Y., Kikuchi, K., and Nagano, T. (2001) *J. Neurochem.* 76, 1404–1410
8. Kuo, R. C., Baxter, G. T., Thompson, S. H., Stricker, S. A., Patton, C., Bonaventure, J., and Epel, D. (2000) *Nature* 406, 633–636
9. Goetz, R. M., Thatte, H. S., Prabhakar, P., Cho, M. R., Michel, T., and Golan, D. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2788–2793
10. Igarashi, J., Thatte, H. S., Prabhakar, P., Golan, D. E., and Michel, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 12583–12588
11. Brown, L. A., Key, B. J., and Lovick, T. A. (1999) *J. Neurosci. Methods* 92, 101–110
12. Yoshioka, T., Iwamoto, N., Tsukahara, F., Irie, K., Urakawa, I., and Muraki, T. (2000) *Br. J. Pharmacol.* 129, 1530–1535
13. Choi, Y.-B., Tenneti, L., Le, D. A., Ortiz, J., Bui, G., Chien, H.-S. V., and Lipton, J. (2000) *Am. J. Respir. Cell Mol. Biol.* 23, 175–181
14. Willmott, N. J., Wong, K., and Strong, A. J. (2000) *J. Neurosci.* 20, 1767–1779
15. Lopez-Figueroa, M. O., Cisamano, C., Morano, M. I., Ronn, L. C., Akil, H., and Watson, S. J. (2000) *Biochem. Biophys. Res. Commun.* 272, 129–133
16. Gorbunov, N. V., Pogue-Geile, K. L., Epperly, M. W., Bigbee, W. L., Draviam, V. V., Watson, B., and soprano, M. A. (2000) *Anal. Chem.* 72, 707–708
17. Berkels, R., Dachs, C., Roesen, R., and Klaus, W. (2000) *Cell Calcium* 27, 281–286
18. Li, D., Shirakami, G., Zhou, X., and Juhano, R. A. (2000) *Am. J. Respir. Cell Mol. Biol.* 23, 175–181
19. Broillet, M. C., Randin, O., and Chatton, J. Y. (2001) *FEBS Lett.* 491, 227–232
20. Noble, D. R., and Williams, D. L. H. (2001) *J. Chem. Soc. Perkin Trans. II*, 15–17
21. Singh, R. J., Hogg, N., Joseph, J., and Kalyanaraman, B. (1995) *FEBS Lett.* 360, 47–51
22. Bers, D. M., Patton, C. W., and Nuccitelli, R. (1994) *Methods Cell Biol.* 40, 3–29
23. Hart, T. W. (1985) *Tetrahedron Lett.* 26, 2013–2016
24. Gunasekar, P. G., Kanthasamy, A. G., Borowitz, J. L., and Isom, G. E. (1995) *J. Neurosci. Methods* 61, 15–21
Orthogonality of Calcium Concentration and Ability of 4,5-Diaminofluorescein to Detect NO
Noriyuki Suzuki, Hirotatsu Kojima, Yasuteru Urano, Kazuya Kikuchi, Yasunobu Hirata and Tetsuo Nagano

J. Biol. Chem. 2002, 277:47-49.
doi: 10.1074/jbc.M108195200 originally published online October 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108195200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 3 of which can be accessed free at http://www.jbc.org/content/277/1/47.full.html#ref-list-1
Additions and Corrections

Vol. 277 (2002) 47–49

Orthogonality of calcium concentration and ability of 4,5-diaminofluorescein to detect NO.

Noriyuki Suzuki, Hirotatsu Kojima, Yasuteru Urano, Kazuya Kikuchi, Yasunobu Hirata, and Tetsuo Nagano

Page 47, footnote 1, last line, and page 49, left column, line 17: The compound “dichlorofluorescin (DCFH)” was misspelled as “dichlorofluorescein.” These are distinct chemical compounds. The correct compound here is “dichlorofluorescin.”