Nitric oxide production in living neurons is modulated by sphingosine: a fluorescence microscopy study

Paola Viani*, Paola Giussani, Anita Ferraretto, Alessandra Signorile, Laura Riboni, Guido Tettamanti

Department of Medical Chemistry and Biochemistry, Study Center for the Functional Biochemistry of Brain Lipids, University of Milan, via F.lli Cervi 93, LITA 20090 Segrate, Milan, Italy

Received 12 July 2001; revised 28 August 2001; accepted 29 August 2001

First published online 21 September 2001

Edited by Felix Wieland

Abstract An investigation was carried out into the possible effect of sphingosine (Sph) on nitric oxide (NO) production in living neurons. Differentiated granule cells were used in a dynamic videoimaging analysis of single cells labeled, simultaneously, with FURA-2 and the NO indicator 4,5-diaminofluorescein. The results demonstrate that Sph exerts a potent inhibitory effect on the Ca^{2+}-dependent production of NO, without modifying the $[Ca^{2+}]_i$. The effect appears to be specific as neither ceramide nor Sph-1-phosphate had any effect on the NO and $[Ca^{2+}]_i$ levels. The data demonstrate that Ca^{2+}-dependent NO production is a specific Sph target in living granule cells, suggesting that this bioactive sphingoid plays a relevant role in neuronal NO signaling.

Key words: Nitric oxide; Sphingosine; Cerebellar granule cell; Dynamic videoimaging; Fluorescence indicator; 4,5-Diaminofluorescein

1. Introduction

Substantial evidence supports a role for nitric oxide (NO) in regulating neuronal functions in both physiological and pathological events such as neurotransmission, brain development, synaptic plasticity, neurodegeneration and neuronal death [1–3]. This implies a tight regulation of NO tissue levels, and owing to the physico-chemical properties of the molecule such a regulation would be based on the control of enzymes involved in NO biosynthesis. Constitutive neuronal NO synthase (nNOS) depends on Ca^{2+}-calmodulin activation, thus the amount of NO produced is governed primarily by intracellular Ca^{2+} levels. Other regulatory mechanisms can operate at the level of Ca^{2+}-calmodulin activation [4,5], occur through phosphorylation/dephosphorylation processes [6] or involve protein–protein interactions [2]. Furthermore, changes in the pathophysiological cell conditions [7,8] could promote a differential regulation of nNOS, thus contributing to the multifunctional role of NO in the nervous system.

Many reports emphasize the role of NO in regulating neuronal growth arrest and differentiation, and indicate an increase in nNOS activity as a characteristic feature of differentiated neurons, including cerebellar granule cells [9–12]. Other bioactive compounds, such as the sphingoid molecules D-erythro-sphingosine (Sph), ceramide (Cer), Sph-1-phosphate (Sph-1P), are also implicated in the control of cell growth and neuronal differentiation [13–15]. In granule cells, for example, neuronal differentiation in culture is characterized by an increase in Cer and Sph levels [16]. This evidence suggests interplay between NO-mediated signaling and sphingoid-mediated signaling in the nervous system.

Prompted by our recent data demonstrating that Sph exerts a specific inhibitory effect on partially purified nNOS [17], our study has been aimed at investigating the possible effect of this sphingoid molecule on NO production in living granule cells. This was achieved through the direct visualization of NO production in living cells using 4,5-diaminofluorescein (DAF-2), a NO-specific fluorescent indicator already proven to be specific and reliable in evaluating NO production in living cells [18–20]. This approach has allowed us to demonstrate that Sph does, in fact, act as a potent inhibitor of Ca^{2+}-dependent NO production in living neurons.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade. L-Arginine, HEPES, EGTA, Cer from bovine brain sphingomyelin, *N*-hexanoyl-D-erythro-sphingosine (C6-Cer), Sph, Sph-1P were all purchased from Sigma; N^G-monomethyl L-arginine monoacetate (L-NMMA) from Alexis Co.; ionomycin, FURA-2 acetoxymethylester (FURA-2/AM), FURA-2 and DAF-2 diacetate (DAF-2 DA) were obtained from Calbiochem.

2.2. Cell cultures and labeling with FURA-2 and DAF-2

Primary cultures of granule cells were prepared from the cerebella of 8-day-old rats (Sprague–Dawley from Charles River) and cultured on poly-L-lysine-coated glass coverslips as previously described [21]. The cells were used after 8–10 days in culture when they were represented by ≥95% differentiated granule cells. Cell morphology was assessed by phase-contrast microscopy. Differentiated granule cells were rinsed twice with Krebs–Ringer–HEPES (KRH) containing: 125 mM NaCl, 25 mM HEPES, 5 mM KCl, 1.2 mM KH_2PO_4, 6 mM glucose, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4. The cells were loaded with 8 μM DAF-2 DA (in 0.2% dimethylsulfoxide (DMSO)) in KRH for an incubation period of 60 min at 37°C [18]; halfway through this period (i.e. after 30 min) FURA-2/AM (in 0.2%

*Corresponding author. Fax: (39)-2-26423209.
E-mail address: paola.viani@unimi.it (P. Viani).

Abbreviations: Cer, ceramide; C6-Cer, *N*-hexanoyl-D-erythro-sphingosine; DAF-2, 4,5-diaminofluorescein; DAF-2 DA, DAF-2 diacetate; DAF-2T, triazolofluorescein; DMSO, dimethylsulfoxide; FURA-2/AM, FURA-2 acetoxymethylester; KRH, Krebs–Ringer–HEPES; L-NMMA, N^G-monomethyl L-arginine monoacetate; NO, nitric oxide; NOS, nitric oxide synthase; Sph, D-erythro-sphingosine; Sph-1P, sphingosine-1-phosphate

0014-5793/01/$20.00
PII: S0014-5793(01)02880-0

DMSO) was added at 5 μM final concentration [22]. The cells were then rinsed twice with KRH, maintaining them in the last KRH washing for 30 min at room temperature to allow de-esterification of the fluorescent probes. De-esterification was performed in both the absence and presence of 1 mM L-arginine (the NOS substrate); in parallel experiments the NOS inhibitor L-NMMA (3 mM) was added to cells incubated with or without L-arginine. DMSO at the final concentration used was found not to interfere with the measures.

2.3. Measurement of $[Ca^{2+}]_i$ and NO production

$[Ca^{2+}]_i$ and NO production were determined simultaneously at the single cell level using microfluorimetry. After cell double labeling, the coverslip was mounted on a thermostated perfusion chamber (PDMI-2, Harvard Apparatus equipped with TC-202A, Medical System Corp.) and placed on an inverted fluorescence microscope stage (Eclipse TE 200, Nikon) equipped with a 40× oil immersion objective. All measures were performed at 37°C. A field of 30–40 cells was chosen randomly and simultaneous recordings of FURA-2 and DAF-2T (the fluorescent triazolofluorescein produced by NO and DAF-2 reaction) images were made before and after cell treatment, using a fluorescence image acquisition and data analysis system supplied by Applied Imaging (High Speed Dynamic Video Imaging Systems, Quanticell 700). The FURA-2 images were obtained through excitation at 340 and 380 nm, the emission signal being collected from a Nikon B-2A filter (DM 505 nm, BA 520 nm) after background subtraction and averaged over four frames with a CCD camera (Extended ISIS, Photonic Science). The $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nm images obtained every 5 s by calibration performed with external standards of calcium and FURA-2 [23]. DAF-2T images were recorded setting the excitation at 495 nm and collecting the emission signal from the Nikon B-2A filter. Background subtraction and frame averaging were performed as for Ca^{2+} determination.

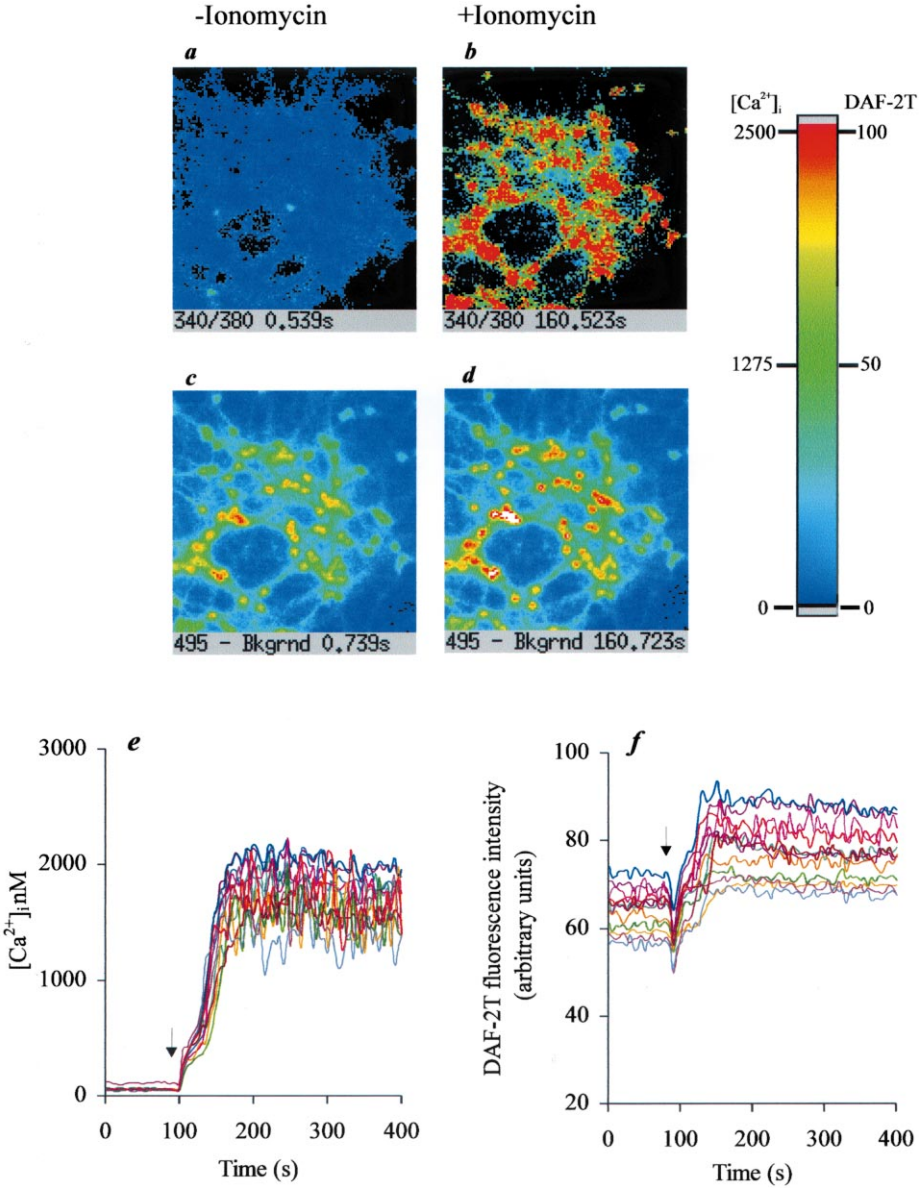

Fig. 1. $[Ca^{2+}]_i$ and NO levels in resting and stimulated granule cells. 340/380 ratio (a,b) and DAF-2T fluorescence (c,d) images of cells doubly labeled with FURA-2 and DAF-2 in the presence of 1 mM L-arginine, before and after ionomycin stimulation. Color changes are representative of different $[Ca^{2+}]_i$ values and DAF-2T fluorescence intensity as indicated in the pseudo-color scale. Single cell kinetic analysis of $[Ca^{2+}]_i$ (e) and DAF-2T fluorescence intensity (excitation 495 nm) (f) in resting conditions and after stimulation with 10 μM ionomycin (indicated by arrow). In e and f the same color represents the kinetic profile of $[Ca^{2+}]_i$ and DAF-2T fluorescence intensity in the same cell. The profiles reported in e and f refer to cells reported in a–d and are representative of at least three different experiments.

2.4. Rationale of experimental design

The resting levels of $[Ca^{2+}]_i$ and NO were determined from the corresponding fluorescence measured in doubly labeled granule cells incubated in 2 ml KRH solution containing or not 1 mM L-arginine, and in the presence or not of 3 mM L-NMMA. In order to increase $[Ca^{2+}]_i$, the cells were treated by the addition of 10 μM ionomycin [24]; this resulted in newly formed NO and caused the recorded fluorescence to rise above the resting levels in the presence of L-arginine, this rise was taken as a measure of authentic Ca^{2+}-dependent NOS activity. Therefore any change in the entity of this rise due to cell stimulation will be the expression of variation in Ca^{2+}-dependent NO production.

An elevation of the intracellular levels of the sphingoid molecules was achieved by adding these molecules to the culture medium under conditions known to prompt rapid incorporation into cells [25–27]. The effect of different sphingoid molecules on $[Ca^{2+}]_i$ levels and NO production was evaluated by administering them (5–10 μM final concentration) to untreated or ionomycin-stimulated cells. Stock solutions of Sph, C6-Cer and Sph-1P were prepared in absolute ethanol, and that of Cer by dissolving it in ethanol/dodecane 98:2 (v/v); the final ethanol and dodecane concentrations never exceeded 0.1% and 0.002%, respectively, at which neither solvent interfered with the measures.

2.5. Other methods

Protein content was assayed according to Lowry et al. [28] using bovine serum albumin as the standard. Statistical significance of differences was determined by the Student *t*-test.

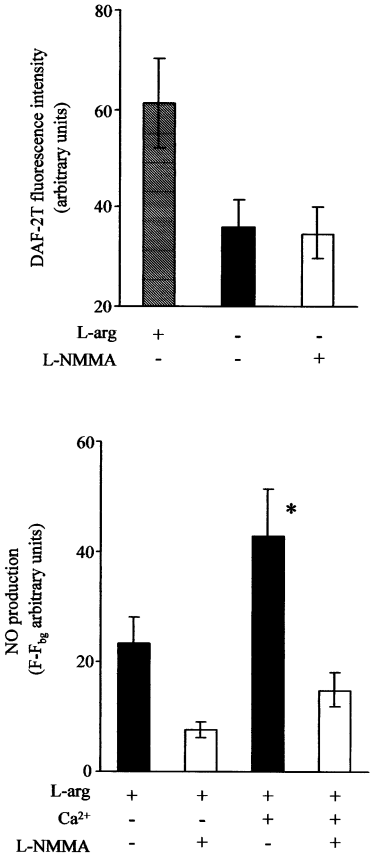

Fig. 2. Effect of L-arginine, L-NMMA and $[Ca^{2+}]_i$ on NO production in granule cells. DAF-2T fluorescence (upper panel) associated with cells and NO production (lower panel) in doubly labeled cells incubated as described in Section 2.4. NO production was evaluated as $F-F_{bg}$ (see Section 3). Data are the mean ± S.D. of values obtained with at least 100 different cells; $^*P \leq 0.001$ Ca^{2+}-stimulated versus resting cells.

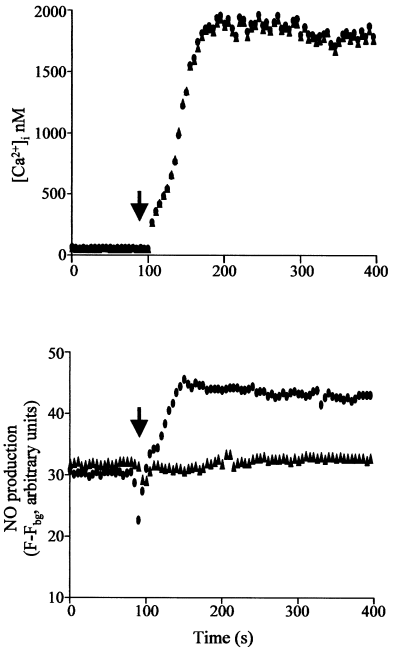

Fig. 3. Effect of Sph on $[Ca^{2+}]_i$ (upper panel) and NO production (lower panel) in differentiated granule cells after stimulation with ionomycin. At the time indicated by the arrow, doubly labeled cells were stimulated with 10 μM ionomycin in the absence (●) or presence of 5 μM Sph (▲). NO production was evaluated as $F-F_{bg}$ as described in Section 3. The curves represent the mean of at least 100 cells, S.D. never exceeding 10% of the mean value.

3. Results

First, we set up experimental conditions enabling the reliable determination of NO production in cultured differentiated granule cells. In Fig. 1a,e it can be seen that in cerebellar granule cells differentiated in culture, simultaneously labeled with FURA-2 and DAF-2 in the presence of L-arginine, the resting concentrations of $[Ca^{2+}]_i$ were about 50 nM. The stimulation of granule cells with 10 μM ionomycin promoted a rapid and sustained $[Ca^{2+}]_i$ increase that reached micromolar concentrations in about 30 s (Fig. 1e). The 340/380 images (Fig. 1a,b) and the kinetic profiles (Fig. 1e) clearly indicate that the behavior of granule cells, with respect to the ionomycin-induced $[Ca^{2+}]_i$ increase, is very homogenous.

As shown in Fig. 1c doubly labeled granule cells incubated in the presence of L-arginine show a cell-associated fluorescence of DAF-2T, even in resting conditions. This basal DAF-2T fluorescence was detectable in all the examined cells. Under these conditions the rise in $[Ca^{2+}]_i$ induced by ionomycin was closely paralleled by a rapid increase in cell-associated DAF-2T fluorescence (Fig. 1d,f), indicating NO formation. All the cells responded homogenously to increased $[Ca^{2+}]_i$, there being an elevation in NO content measured as DAF-2T fluorescence.

To evaluate whether the intensity of DAF-2T fluorescence observed in resting conditions is representative of NO production, we measured cell-associated fluorescence in granule cells incubated in the absence of L-arginine. As shown in Fig. 2 (upper panel) the removal of L-arginine from the incubation medium strongly reduced, but did not abolish, the cell-associated fluorescence detected with excitation at 495 nm. Note, too, that the fluorescence measured in the absence of exoge-

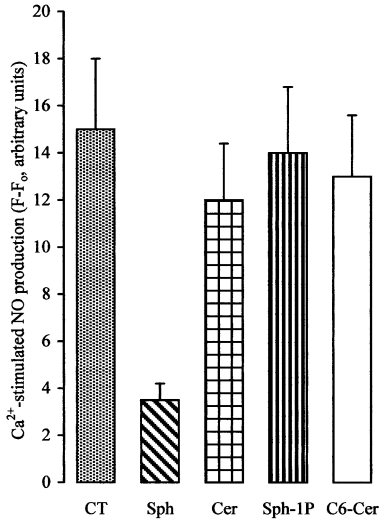

Fig. 4. Effect of different sphingoid molecules on Ca^{2+}-stimulated NO production in cerebellar granule cells. Doubly labeled cells were simultaneously stimulated with 10 μM ionomycin in the absence (CT) or in the presence of 5 μM sphingoid molecule. Ca^{2+}-stimulated NO production was evaluated as $F-F_0$, where F represents the maximal DAF-2T fluorescence intensity monitored after ionomycin stimulation, and F_0 DAF-2T fluorescence intensity in resting conditions. Data are the mean ± S.D. of at least 100 cells. Similar results were obtained at 10 μM sphingoid concentration.

nous L-arginine was insensitive to the presence of the NOS inhibitor L-NMMA (Fig. 2, upper panel). Thus when evaluating cell-associated fluorescence, that was most certainly due to NO production, we assumed a background fluorescence value (F_{bg}) of cells incubated in the presence of L-NMMA and absence of L-arginine. The mean F_{bg} value was found to be 34 ± 3.5 (mean ± S.D., $n = 120$) arbitrary units. Using F_{bg} as the background, the production of NO was measured in basal conditions, i.e. cells incubated with L-arginine (Fig. 2, lower panel). The induced $[Ca^{2+}]_i$ rise resulted in a significant increase in intracellular NO, reaching a value corresponding to 180% the starting level 80 s after ionomycin stimulation (Fig. 2, lower panel). Worth noting is the about 80% inhibition of both basal and $[Ca^{2+}]$-stimulated NO production when the cell incubation was done in the presence of L-NMMA.

These results led us to consider our experimental model sufficiently sensitive and reliable to investigate the effect of Sph on NO production at the single cell level. The administration of Sph along with ionomycin did not modify the kinetic profile of the $[Ca^{2+}]_i$ increase (Fig. 3, upper panel). On the contrary, in all the examined cells, Sph almost completely hindered the Ca^{2+}-dependent production of NO (Fig. 3, lower panel). Conversely, when Cer or Sph-1P were administered with ionomycin to the cells there appeared to be no modification of either the $[Ca^{2+}]_i$ increase (data not shown) or Ca^{2+}-dependent NO production (Fig. 4). Also C6-Cer, the cell-permeable analog of Cer, was ineffective. Finally, the kinetic

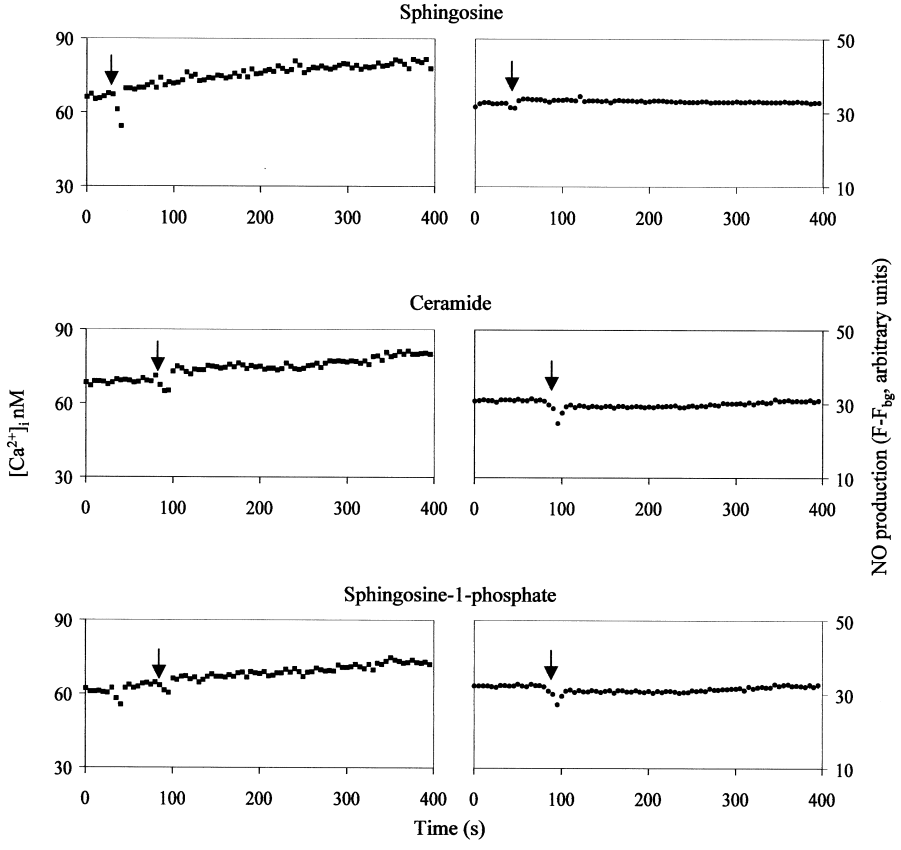

Fig. 5. Effect of different sphingoids on the kinetic profiles of $[Ca^{2+}]_i$ (left panels) and NO production (right panels) in resting granule cells. At the time indicated by the arrow, cells were stimulated with 5 μM of different sphingoids in the absence of ionomycin. All curves represent the mean of at least 100 cells, S.D. never exceeding 10% of the mean.

profiles of $[Ca^{2+}]_i$ and intracellular NO (Fig. 5) indicate that stimulation of cerebellar granule cells differentiated in culture with different sphingoids in the absence of ionomycin leads to no modification of either the $[Ca^{2+}]_i$ or intracellular NO levels.

4. Discussion

In the present study we performed a videoimaging analysis of cerebellar granule cells differentiated in culture, doubly labeled with FURA-2 and DAF-2. The approach has enabled us to measure intracellular calcium levels and NO production simultaneously in the same cell, and provides the possibility of following, in the same experiment, $[Ca^{2+}]_i$ and NO variations in neuronal cells.

The results obtained by FURA-2/DAF-2T videoimaging analysis have allowed us to demonstrate that NO is already detectable in resting conditions, even when $[Ca^{2+}]_i$ is as low as 50 nM. In fact, the fluorescence intensity measured in resting cells depends on the presence of arginine in the incubation medium, and is strongly inhibited by the presence of the NOS inhibitor L-NMMA. These basal levels of NO could be explained as being due to the action of a Ca^{2+}-independent NOS previously demonstrated to occur in differentiated granule cells [11]. Moreover, single cell analysis demonstrated that in all the analyzed cells the NO production paralleled the ionomycin-induced $[Ca^{2+}]_i$ increase. This indicates differentiated granule cells to have very homogenous behavior where Ca^{2+}-calmodulin-dependent nNOS is concerned, which is in agreement with the notion that the expression of this enzyme is a specific feature of these cells [11].

The evidence that NO production can be evaluated by dynamic videoimaging analysis of DAF-loaded cells led us to investigate the possible regulatory role of Sph on NOS activity in living neurons. Our results demonstrate that Sph strongly and specifically reduces the Ca^{2+}-dependent NO production in these cells, suggesting an important impact of this bioactive sphingoid on the nNOS signaling pathway. In fact the capacity of Sph to inhibit Ca^{2+}-dependent NOS does not appear to be due to this molecule's direct effect on $[Ca^{2+}]_i$ since Sph does not modify the basal levels of $[Ca^{2+}]_i$ or the ionomycin-stimulated Ca^{2+} influx into these cells. In previous in vitro studies we demonstrated that Sph inhibits the Ca^{2+}-calmodulin activation of the enzyme by a competitive mechanism [17], thus it can be hypothesized that also in living cerebellar granule cells Sph interferes with the Ca^{2+}-calmodulin-dependent activation of nNOS.

The effect of Sph appears to be specific since neither Sph-1P nor Cer, the two bioactive Sph metabolites, has any influence on Ca^{2+}-calmodulin-dependent NOS activity. Thus it can be excluded that the effects observed with Sph are due to the very rapid metabolic processing of Sph known to occur in differentiated granule cells [29]. It should also be noted that the stimulation of cerebellar granule cells with Sph, Cer or Sph-1P, neither modified $[Ca^{2+}]_i$, nor influenced the basal NO levels. This indicates that (a) Sph exerts its regulatory effect on the Ca^{2+}-dependent, but not on the basal, production of NO; (b) Cer, unlike the observations for endothelial NOS [30], does not promote any Ca^{2+}-independent activation of nNOS; (c) Sph-1P does not affect $[Ca^{2+}]_i$, unlike what occurs in other cell types [31].

In conclusion our data demonstrate, for the first time, that in intact neuronal cells, Ca^{2+}-dependent NOS represents a target for Sph, suggesting that, in neurons, this bioactive sphingoid can regulate NO production by nNOS, in antagonism to the control exerted by Ca^{2+} on the same enzyme. In this study the effect of Sph was obtained by administering exogenous Sph at micromolar concentrations, a condition known to rapidly modify Sph levels in granule cells [29]. On the basis of previous data demonstrating a time- and dose-dependent incorporation of exogenous Sph in granule cells [26,29], the amount of exogenous Sph incorporated into the cells within the time of the sphingoid base effect on NO levels can be calculated to be about 100–400 pmol/mg cell protein. This is about 0.5–2-fold the Sph levels of differentiated granule cells [29]. These variations are in the order of magnitude of Sph changes in different cells after stimulation with different extracellular agents [32]. It is tempting to speculate that any change in the intracellular Sph levels consequent to specific stimuli [33] could modulate the response of nNOS to Ca^{2+}-calmodulin activation, representing a new intersignaling mechanism with the potential to modulate nNOS activity independently of the wide spectrum of cell functions regulated by $[Ca^{2+}]_i$. Finally, these results give further support to the existence and functional relevance of 'cross-talk' between NO- and sphingolipid-mediated signal transduction in neuronal cells.

Acknowledgements: This work was supported by grants from the Italian Ministry of University and Research (MURST PRIN 1999 and ex 60% to P.V., MURST PRIN 1997 to G.T. and MURST PRIN 1998, 2000 to L.R.).

References

[1] Dawson, T.M., Sasaki, M., Gonzales-Zulueta, M. and Dawson, V.L. (1998) in: Progr. Brain Res. (Mize, R.R., Dawson, T.M., Dawson, V.L. and Friedlander, M.J., Eds.), Vol. 118, pp. 3–11, Elsevier Science.
[2] Bredt, D.S. (1999) Free Radic. Res. 31, 577–596.
[3] Contestabile, A. (2000) Biochim. Biophys. Acta 32, 476–509.
[4] Khatun, S., Kanayama, N., Belayet, H.M., Sato, E., Tokunaga, N., Kobayashi, T. and Terno, T. (1999) Neuroscience 93, 605–609.
[5] Wolff, D.J., Datto, G.A. and Samtovicz, R.A. (1993) J. Biol. Chem. 268, 9430–9436.
[6] Hu, J. and El-Fakahani, E.E. (1996) Cell Signal. 8, 185–189.
[7] Ovadia, H., Rosenmann, H., Shezen, E., Halimi, M., Ofran, I. and Gabizon, R. (1996) J. Biol. Chem. 271, 16856–16861.
[8] Luo, Z.D., Chaplan, S.R., Scott, B.P., Cizkova, D., Calcutt, N.A. and Yaksh, T.L. (1999) J. Neurosci. 19, 9201–9208.
[9] Munoz-Fernandez, M.A., Cano, E., O'Donnell, C., Doyle, J., Liew, F.Y. and Fresno, M. (1994) J. Neurochem. 62, 1330–1334.
[10] Peunova, N. and Enikopolov, G. (1995) Nature 375, 68–73.
[11] Viani, P., Giussani, P., Riboni, L., Bassi, R. and Tettamanti, G. (1997) FEBS Lett. 408, 131–134.
[12] Cote, F., Laflamme, L., Payet, M.D. and Gallo Payet, N. (1998) Endocr. Res. 24, 403–407.
[13] Futherman, A.H. (1998) Biochemistry (Moscow) 63, 74–83.
[14] Riboni, L., Prinetti, A., Bassi, R., Caminiti, A. and Tettamanti, G. (1995) J. Biol. Chem. 270, 26868–26875.
[15] Riboni, L., Viani, P., Bassi, R., Stabilini, A. and Tettamanti, G. (2000) Glia 32, 137–145.
[16] Tettamanti, G., Prinetti, A., Bassi, R., Viani, P., Giussani, P. and Riboni, L. (1996) J. Lipid Mediat. Cell Signal. 14, 263–275.
[17] Viani, P., Giussani, P., Riboni, L., Bassi, R. and Tettamanti, G. (1999) FEBS Lett. 454, 321–324.
[18] Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H. and Nagano, T. (1998) Anal. Chem. 70, 2446–2453.
[19] Nakatsubo, N., Kojima, H., Kikuchi, K., Nagoshi, H., Hirata,

Y., Maeda, D., Imai, Y., Irimura, T. and Nagano, T. (1998) FEBS Lett. 427, 263–266.
[20] Willmott, N.J., Wong, K. and Strong, A.J. (2000) J. Neurosci. 20, 1767–1779.
[21] Gallo, V., Ciotti, M.T., Coletti, A., Aloisi, F. and Levi, G. (1982) Proc. Natl. Acad. Sci. USA 79, 7919–7923.
[22] Tsien, R.Y. and Poenie, G.M. (1986) Trends Biochem. Sci. 11, 450–455.
[23] Grynkiewicz, G., Poenie, G.M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[24] Oka, M., Itoh, Y., Ukai, Y. and Kimura, K. (1999) J. Neurochem. 72, 1315–1322.
[25] Ji, L., Zhang, G., Uematsu, S., Akahori, Y. and Hirabayashi, Y. (1995) FEBS Lett. 358, 211–214.
[26] Riboni, L., Viani, P., Bassi, R., Giussani, P. and Tettamanti, G. (2000) J. Neurochem. 75, 503–510.
[27] Van Valdhoven, P.P. and Mannaerts, G.P. (1994) Biochem. J. 299, 597–601.
[28] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[29] Riboni, L., Bassi, R., Prinetti, A., Viani, P. and Tettamanti, G. (1999) Biochem. J. 338, 147–151.
[30] Igarashi, J., Thatte, H.S., Prabhakar, P., Golan, D.E. and Michel, T. (2000) Proc. Natl. Acad. Sci. USA 96, 12583–12588.
[31] Spiegel, S. and Milstien, S. (2000) Biochim. Biophys. Acta 1484, 107–116.
[32] Riboni, L., Viani, P., Prinetti, A., Bassi, R. and Tettamanti, G. (1997) Progr. Lipid Res. 36, 153–195.
[33] Spiegel, S., Foster, D. and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 159–167.