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Short Communication

Calcium/calmodulin dependence of nitric oxide synthase from *Viviparus ater* immunocytes

D Tagliazucchi, A Conte

Department of Agricultural Sciences, University of Modena and Reggio Emilia, Italy

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Abstract

The calcium ion dependence of soluble and particulate nitric oxyde synthase (NOS) activity from *Viviparus ater* immunocytes was investigated. At a calcium ion concentration of 2 nM, the NOS activity measured by citrulline formation was 27.1 ± 2.2 and 9.3 ± 0.8 pmol/min/10^6cell for soluble and particulate NOS, respectively. The increase in free calcium ion concentration to 300 nM increases enzyme activity to 57.5 ± 4.1 and 23.5 ± 1.2 pmol/min/10^6cell, respectively. The 50 % activation of the calcium-dependent activity is 91 and 97 nM Ca^{2+} for soluble and particulate enzymes. Trifluoperazine, an inhibitor of the calmodulin-dependent enzyme, partially inhibits both activities. Soluble NOS is five times more sensitive than particulate NOS. The behaviour of both activities with three NOS inhibitors (7-nitroindazole, S-methylisothiourea sulphate, diphenyleneiodonium) is very similar, with IC_{50} values that are not significantly different. The calcium ion dependence of NOS activities, in a range of free calcium ion variations, which are transiently observed in receptor-stimulated cells, suggests that nitric oxyde in *V. ater* immunocytes not only has a defensive role but also signalling relevance in cross-talking between immunocytes and other cells.

Key words: mollusc; *Viviparus ater*; immunocytes; nitric oxide synthase; calcium ion dependence

Introduction

Immunocytes are the cells of the immune response in molluscs and other invertebrates against not-self materials. Recognition, phagocytosis and killing of virus and bacteria are one of the most important functions of invertebrate immunocytes (Ottaviani, 1992).

In mammalian phagocytic cells, oxygen reactive species (ROS), such as superoxide ions and hydrogen peroxide, hypochlorous acid and nitric oxide (NO), are produced to kill phagocyted organisms. From NO and superoxide ions, the more reactive peroxynitrite ion and other oxygen radicals are formed (Beckman et al., 1990; Porasuphatana *et al*., 2001; Heales *et al*., 1999). The first evidence for NO production and utilisation as a bactericidal agent by invertebrate mollusc immunocytes was reported by Ottaviani *et al*. (1993). The nitric oxide synthase (NOS) activity of immunocytes of the fresh water snail *Viviparus ater* has been partially characterised biochemically (Conte and Ottaviani, 1995).

NOS activity shows a partial dependence on calcium ions. It is also present in particulate fractions and is induced by lipopolysaccharides (LPS) (Conte and Ottaviani, 1995).

Three main different forms of NOS have been isolated from different cell types: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) from macrophages (Pollock *et al*., 1991; Lamas *et al*., 1992; Bredt *et al*., 1991; Stuehr *et al*., 1991). The constitutive eNOS and nNOS have an absolute requirement for calcium ions and calmodulin. eNOS is mainly, if not completely, membrane associated. The presence of covalently-bound myristoyl and palmitoyl residues on protein molecules have an important role in binding to membranes. nNOS is mainly cytosolic, but may be bound to cell membranes. iNOS is cytosolic and is expressed upon immunological and inflammatory stimulation. This form is independent of calcium ions and calmodulin (Cho *et al*., 1992; Steven-Truss and Marletta, 1995). iNOS may produce toxic and lethal NO concentrations.

Corresponding Author:
Davide Tagliazucchi
University of Modena and Reggio Emilia, Department of Agricultural Sciences, Via Kennedy 17, 42100 Reggio Emilia, Italy
E-mail: tagliazucchi.davide@unimore.it

The NO produced by nNOS and eNOS has a signalling role under the strict control of intracellular calcium ions. Alternative spliced forms of NOS have been demonstrated (Silvagno et al., 1996; Magee *et al*., 1996). The NOS enzyme(s) from immunocytes of *V. ater* combine the properties of different NOSs (Conte and Ottaviani, 1995) and cannot be identified with any of the enzymes studied so far, in particular the iNOS of mammalian phagocytic cells. In the immunocytes of *Mytilus edulis,* morphine induces a transient increase in intracellular calcium ions, followed by NO release (Nieto-Fernandez *et al*., 1999) suggesting that the immunocytes of this invertebrate have NOS with properties that are similar to those of *V. ater*.

The aim of this study was the characterisation of calcium ion dependence of soluble and particulate NOS of the snail *V. ater*.

Materials and methods

Reagents

(6R)–5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and L-[2,3,4,5-^{3}H]arginine monohydrochloride (58 Ci/mmol) from Amersham (Buckinghamshire, England). The ion-exchange resin AG50WX-8 was supplied by Bio-Rad (Milan, Italy). Calmodulin, glucose-6-phosphate dehydrogenase from yeast (EC 1.1.1.49) and glucose-6-phosphate were from Serva (Heidelberg, Germany). 7-nitroindazole (7-NI), S-methylisothiourea sulphate (SMT), diphenyleneiodonium (DPI), and trifluoperazine (TFP) were from Calbiochem (Darmstadt, Germany). Nitrate reductase from *Aspergillus* spp. (EC 1.6.6.2), lipopolysaccharide (LPS) from *Escherichia coli*, N^G-monomethyl-L-arginine (L-NMMA) and all other biochemicals were obtained from Sigma (Milan, Italy).

Snails

Adult specimens of *Viviparus ater* were collected from a freshwater canal near Modena (Italy), in spring and early summer. The animals were maintained at room temperature in de-chlorinated freshwater, for at least a week before the experiments. Snail haemolymph was obtained by prodding the animal's foot and collected with a Pasteur pipette. Immunocytes were obtained by centrifugation at 600 x g. The cells were washed twice with snail saline solution (Ottaviani, 1983), counted and collected using centrifugation.

Determination of NOS activity

Immunocytes were homogenised with Ultra-Turrax (IKA-WERK, Germany) in 5 vols. of ice-cold solution, containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1mM dithiothreitol (DTT), 1mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml antipain and 10 μg/ml bestatin, brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 20,000 x g for 30 min at 4 °C, and the supernatant was freed from low molecular mass compounds by Sephadex G-25 chromatography (Werner-Felmayer *et al*., 1993). The protein fraction was eluted with a buffer containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT and the above-mentioned protease inhibitors.

NOS activity was assayed by following the conversion of radiolabelled arginine to citrulline. Standard reaction mixtures contained 50 mM HEPES, pH 7.4, 0.5 mM EDTA, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NADPH, 1 mM DTT, 12 mM L-valine, 1 mM citrulline, a variable amount of L-arginine, 80,000-100,000 cpm of purified L-[2,3,4,5-^{3}H]arginine monohydrochloride and 5-50 μl of Sephadex G-25 eluate in a final volume of 100 μl. After 30 min incubation at 37 °C, [^{3}H]citrulline was quantified by liquid scintillation counting, after separation from [^{3}H]arginine by cation exchange (AG50WX-8) (Bredt and Snyder, 1989). The NOS activity of the non-soluble fraction was determined by washing the immunocyte pellet twice with PBS, centrifuging at 20,000 x g for 30 min at 4 °C and re-suspending with the buffer used for Sephadex G-25 chromatography. NOS activity was determined by calculating the difference between the [^{3}H]citrulline produced in the presence and absence of 10 mM N^G-monomethyl-L-arginine (L-NMMA, an inhibitor of mammalian NOS) in a standard reaction mixture. Activity was calculated using the radiochemical method and expressed as pmol of [^{3}H]citrulline formed/min/10^6 cells. When calcium ion dependence was investigated, variable amounts of $CaCl_2$ and EGTA were added to the reaction mixture.

Free calcium concentrations were calculated following Fabiato (1988) and controlled by the FURA 2 method, in accordance with Mülsch et al. (1989).

In some cases, incubations also contained variable concentrations of the NOS inhibitors: 7-NI, SMT, DPI or TFP.

The results are expressed as means ± SD of at least three independent experiments performed in triplicate. Proteins were determined in accordance with the modified Lowry method (Markwell et al, 1981), with serum albumin as standard.

Results

The dependence of soluble and particulate NOS activities on free calcium ion concentrations at pH 7.4 is reported in Fig. 1. The soluble enzyme activity (upper line) at a free Ca^{2+} concentration of 2 nM was 27.1 ± 2.2 pmol/min/10^6 cells and progressively increased to 57.5 ± 4.1 at 300 nM Ca^{2+}. The enzyme activity slightly decreased at higher calcium concentrations. The particulate NOS activity (lower line), which is about 40 % of the soluble activity, showed similar calcium dependence. Fifty percent activation is obtained at a 91 and 97 nM calcium ion concentration for soluble and particulate enzymes, respectively. Calcium ions activated both soluble and particulate NOS enzymes at concentrations that are physiologically observed during a transient calcium ion increase in cells stimulated by signalling molecules.

The calmodulin dependence, evaluated by trifluoperazine inhibition of NOS activity, is reported in Fig. 2. Trifluoperazine is a calmodulin antagonist that inhibits Ca^{2+}/calmodulin-dependent enzymes. The NOS soluble activity (upper line), measured at a free Ca^{2+} concentration of 300 nM, decreased with increasing concentration of TFP to 55 % of the

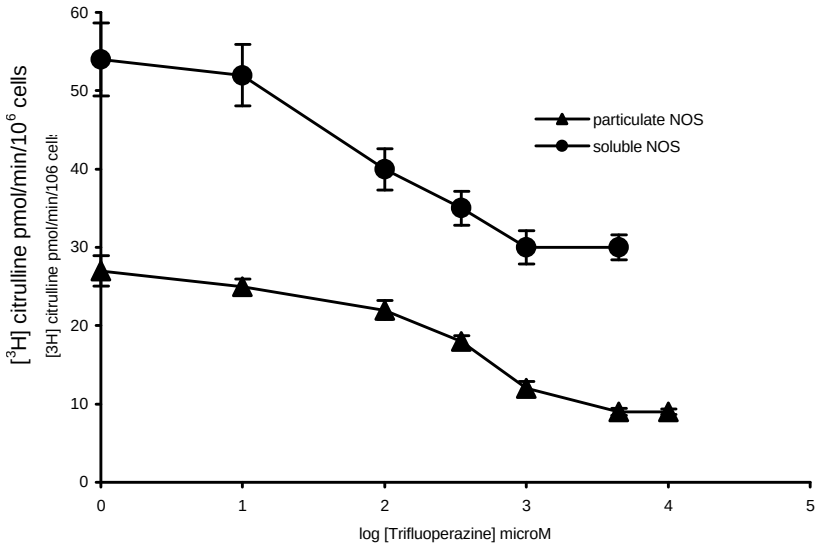

Fig. 1 Dependence of soluble and particulate NOS activity on free Ca^{2+} concentration. Each point is the mean ± SD of three independent experiments performed in triplicate.

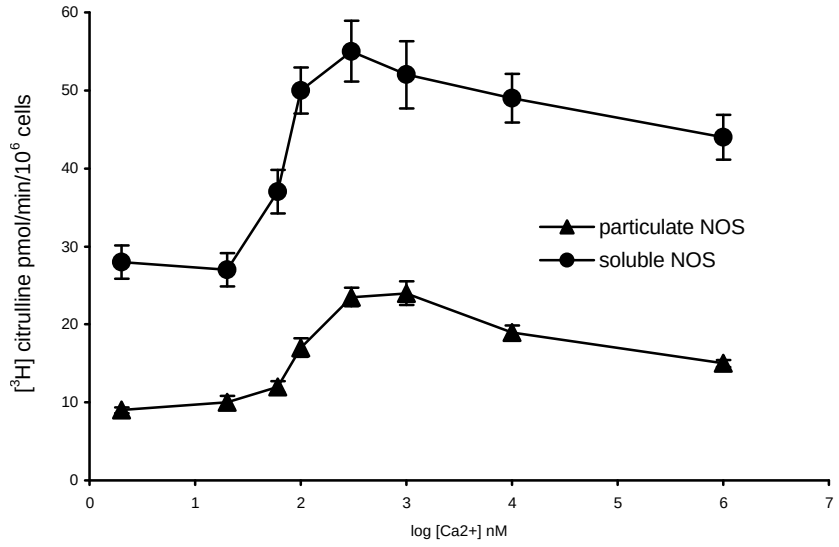

Fig. 2 Dependence of soluble and particulate NOS activity on trifluoperazine concentration. Each point is the mean ± SD of three independent experiments performed in triplicate.

Table 1 IC_{50} values of some NOS activity inhibitors. The inhibitors were included in standard reaction mixtures, containing 1 μM arginine and prepared as described in the text. The data are the means ± SD of three independent experiments performed in triplicate.

	Soluble NOS	Particulate NOS
TFP (μM)	74 ± 20	383 ± 92
7-NI (nM)	715 ± 134	648 ± 118
DPI (nM)	102 ± 29	130 ± 26
SMT (nM)	877 ± 190	1051 ± 276

activity of the control at about 1 mM TFP. No further decrease in the enzyme activity was observed at higher TFP concentrations. The particulate bound activity (lower line) was inhibited by TFP at about a 5-times higher concentration than soluble NOS (Table 1) similarly to what we found with the carp enzyme (Conte and Ottaviani, 1998). Table 1 reports the IC_{50} values of the three other inhibitors tested. No significant difference in the IC_{50} values was observed between the soluble and particulate enzymes.

Discussion

We found that calcium and calmodulin dependence, evaluated by TFP inhibition, of NOS activity from immunocytes of *V. ater,* measured by citrulline formation, is similar to those of the mutant Ä 45 of the human eNOS enzyme (Chen and Wu, 2003). In this mutant, residues 594-606 and 614-645 of human eNOS are removed. These residues are not present in human and mouse macrophage iNOS. The Ä 45 mutant is able to form NO, measured by citrulline formation, in the absence of calmoduline or calcium ions, at 60% of the rate in their presence. It has been suggested by Roman *et al.* (2000) that the Ä 45 segments play a relevant role in Ca^{2+}/calmodulin dependence. The presence of these segments decreases or inhibits the electron flow from the reductase domain, which binds NADPH, to the oxygenase domain, which binds arginine. Ca^{2+}/calmodulin, binding to the eNOS or nNOS, remove inhibition and allow electron transfer from reductase to oxygenase. Geller *et al.* (1993) reported the molecular cloning and expression in 293 embryonic kidney cells of NOS induced by LPS in human hepatocytes. The chelating agents EDTA and EGTA decreased the activity of this enzyme by 30 % but failed to obtain complete inhibition. Trifluoperazine decreased activity by 50 %. The amino acid sequence reported by Geller *et al.* (1993) appears to be the same as NOS from human macrophages. Whatever the molecular mechanism of partial calcium dependence of *V. ater,* NOS raises several questions on its roles and regulation in immunocytes.

NOS has been demonstrated in the nervous system of several species of snail. NO has neurotransmitter-like functions, for example, it is necessary for the transmission of sensory information to the central pattern generator for feeding behaviour (Elphick *et al*., 1995) and the activation of buccal motor patterns (Moroz *et al*., 1993). NO is involved in neural transmission to intestinal muscles in *Helix lucorum* but enteric release of NO is blocked during snail dormancy (Roszer *et al*., 2004). NO has a role in developing the nervous system of the snail *Ilyanassa obsoleta* (Thavaradhara and Leise, 2001) and regulating the early embryonic behaviour in the snail *Helisoma trivolvis* (Cole *et al*., 2002).

In all of the above examples, the constitutive calcium-dependent nNOS is considered to be the enzyme that produces NO. Identification of NOS enzymes has been carried out mainly by immunocytochemical methods, using antibodies to mammalian nNOS. Amino acid sequence studies show that neuronal NOS from insects (*Drosophila melanogaster* and *Schistocerca gregaria*) and the snail *Lymnaea stagnalis,* share 43-67 % identity with mammalian nNOS (Ogunshola *et al*., 1995; Regulski and Tully, 1995). It has been reported that *V. ater* soluble NOS is inhibited by 70 % by mammalian nNOS antibodies (Conte and Ottaviani, 1995).

Moroz *et al.* (1996) demonstrated constitutive calcium-independent NOS activity in the nervous system of some molluscan species, which is largely associated with particulate fractions. Using immunochemical investigations, Xie *et al.* (2002) showed that the substrate arginine and the NOS enzyme are localised in the separate, but adjacent, neurons of the snail *Helix pomatia*. The product citrulline is observed in the neurons, which contain the NOS enzyme, suggesting an unknown signalling pathway between neurons to maintain arginine and NO homeostasis.

The examples reported above show the complexity of the properties and regulation of NOS activity in neuronal cells of snails, as well the expanding roles of NO.

The defense role of snail immunocyte NOS has been demonstrated (Ottaviani *et al*., 1993). During phagocytosis, these cells also produce oxygen species that may combine with NO to form the more reactive peroxynitrite, increasing killing capacity. However, it seems that immunocytes are not only of immunological relevance, as they also synthesise signalling peptides (Ottaviani *et al*., 1992). Invertebrate immunocytes respond to multiple signal molecules that form the immunoregulatory network. Cytokine-like factors affect immune functions such as cell motility, chemotaxis, phagocytosis and cytotoxicity in invertebrate immunocytes and are able to induce NO (Ottaviani *et al*., 2004). Cytokine-stimulated immunocytes in *M. edulis* modulate ganglionic NO release, which later affects their activity level, demonstrating that ganglionic NO is involved in down-regulating immunocyte activity (Stefano *et al*., 2004). It has been demonstrated that *M. edulis* immunocytes have a μ_3 morphine receptor, coupled to NO release in an intracellular calcium-mediated manner (Nieto-Fernandez *et al*., 1999). NO release may mediate morphine-induced changes in immunocyte conformation, lowering chemotactic activity, cellular velocity, and adherence (Stefano *et al*., 1993). Furthermore, naturally occurring cannabinoids may share the NO-producing effector system with opiate alkaloids in these cells (Stefano *et al*., 1996). Not only can morphine-like and

cannabinoid substances be found in invertebrate immunocytes, there is also a morphine-like precursor that provides proof of the presence of opiates in these animals (Stefano ***et al.***, 1993). This evidence, supporting the complexity of the immunoregulatory processes, enables us to assume that the binding of signalling molecules to *V. ater* immunocyte receptors may determine a calcium-dependent release of NO, which allows complex and physiologically relevant cross-talking between immunocytes and other cells in molluscs.

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