ELSEVIER

Brain Research 924 (2002) 82–89

BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

The biochemical substrate of nitric oxide signaling is present in primitive non-cognitive organisms

George B. Stefano[a,*], Enzo Ottaviani[b]

[a]*Neuroscience Research Institute, State University of New York College at Old Westbury, Old Westbury, NY 11568-0210, USA*
[b]*Department of Animal Biology, University of Modena and Reggio Emilia, via Campi 213/D, 41100 Modena, Italy*

Accepted 16 October 2001

Abstract

Nitric oxide has been shown to have diverse actions in the mammalian nervous, immune and vascular systems. These include antimicrobial and antiviral activities as well as the modulation of cell adherence. In the nervous system, nitric oxide modulates neurotransmitter release, neurosecretion and behavioral activities such as feeding. In the present review, we discuss the finding that invertebrate organisms also contain nitric oxide and that they appear to use this multidimensional molecule in a similar manner as noted for mammals. Therefore, nitric oxide signaling appears to have emerged first in these primitive non-cognitive organisms. We conclude that basal nitric oxide functioning was established in these organisms and that this molecule was later employed in man, including its involvement in cognitive neural processes.

Theme: Neural basis of behavior

Topic: Cognition

Keywords: Cognition; Nitric oxide; Invertebrate; Morphine; Cannabinoid; Nitric oxide synthase

1. Introduction

Our studies demonstrate considerable conservation in cannabinoid and opiate/opioid processes between invertebrates and humans. Historically, selected invertebrate preparations, most notably the squid giant axon, snail brain, cray fish neuromuscular junction and *Limulus* eye, have proven invaluable for the study of basic neurobiological phenomena. Known evolutionary relationships, the presence of many of the same mediators and transmitters in their nervous systems, and the evidence for the presence of opiate processes, specifically in molluscs and in the leech [54–56,60,61,74], make these organisms ideal for the study of these processes. Recently, these conserved signaling systems have been extended to include nitric oxide (NO).

Nitric oxide, generated by nitric oxide synthase (NOS) through the deamination of arginine, is a gaseous free radical that, as an intracellular second messenger and signaling or cytotoxic molecule, intervenes in several physiological processes [41]. The presence and the biological role of NO in invertebrates have only recently been addressed. The activity of NADPH diaphorase, an enzyme known to be identical to mammalian brain and peripheral NOS [9,26], has been demonstrated in the nervous tissue of the more advanced invertebrates with well-organized systems [14]. Moreover, biochemical studies in insects have shown the presence of Ca^{2+}/calmodulin-stimulated NOS activity and NO-activated cGMP [4], suggesting that the NO/cGMP signaling pathway is common both in invertebrate and vertebrate nervous systems. In the insect *Manduca sexta*, the communication between olfactory receptor neurons and projection neurons of the antennal lobe may be regulated by the NO/cGMP signaling pathway [45]. A NO/cGMP pathway has also been reported in the nervous system of the mollusc *Helix pomatia* [27], and in the mollusc *Aplysia californica*, it is thought that NO is involved in interneural communication [28]. Moreover, in

*Corresponding author. Tel.: +1-516-876-2732; fax: +1-516-876-2727.

E-mail address: gstefano@li.net (G.B. Stefano).

0006-8993/02/$ – see front matter
PII: S0006-8993(01)03227-9

the protocerebral lobe of the mollusc *Limax maximus*, NO mediates the network oscillations of olfactory interneurons [22], and the NO-containing neurons of the buccal ganglia of the mollusc *Lymnaea stagnalis* seem involved in the coordination of the feeding program [42]. Another proposed role of NO is its involvement in neural plasticity that underlies processes of learning and memory [14].

The presence and the activity of NOS were also found in the immune system of invertebrates by histochemical, immunocytochemical and biochemical approaches (Fig. 1). In particular, NADPH diaphorase activity was detected in immunocytes of the arthropod *Limulus polyphemus* [51], and the mollusc *Viviparus ater* [20]. Activation of molluscan [20] by bacteria, lipopolysaccharide (LPS) or silica beads induced NOS activity, and inhibitors such as L-NMA and L-NMMA were able to block this enzymatic activity. The presence of NOS was also demonstrated in molluscan immunocytes with an anti-NOS polyclonal antibody, and after bacterial stimulation an increased immunoreactivity was observed, suggesting that immunocyte NOS is inducible [20] (Fig. 1).

The biochemical characterization of NOS, performed in naive and LPS-stimulated molluscan immunocytes [7], demonstrated that the enzyme activity was comparable to that of mammalian cells. Indeed, a close relationship between [^{3}H]citrulline and nitrite/nitrate formation has been found, and the reaction was inhibited with the competitive inhibitor L-NMMA. In addition, the presence of NADPH was required for full activity of the enzyme. A polyclonal rabbit antiserum to the C-terminal synthetic

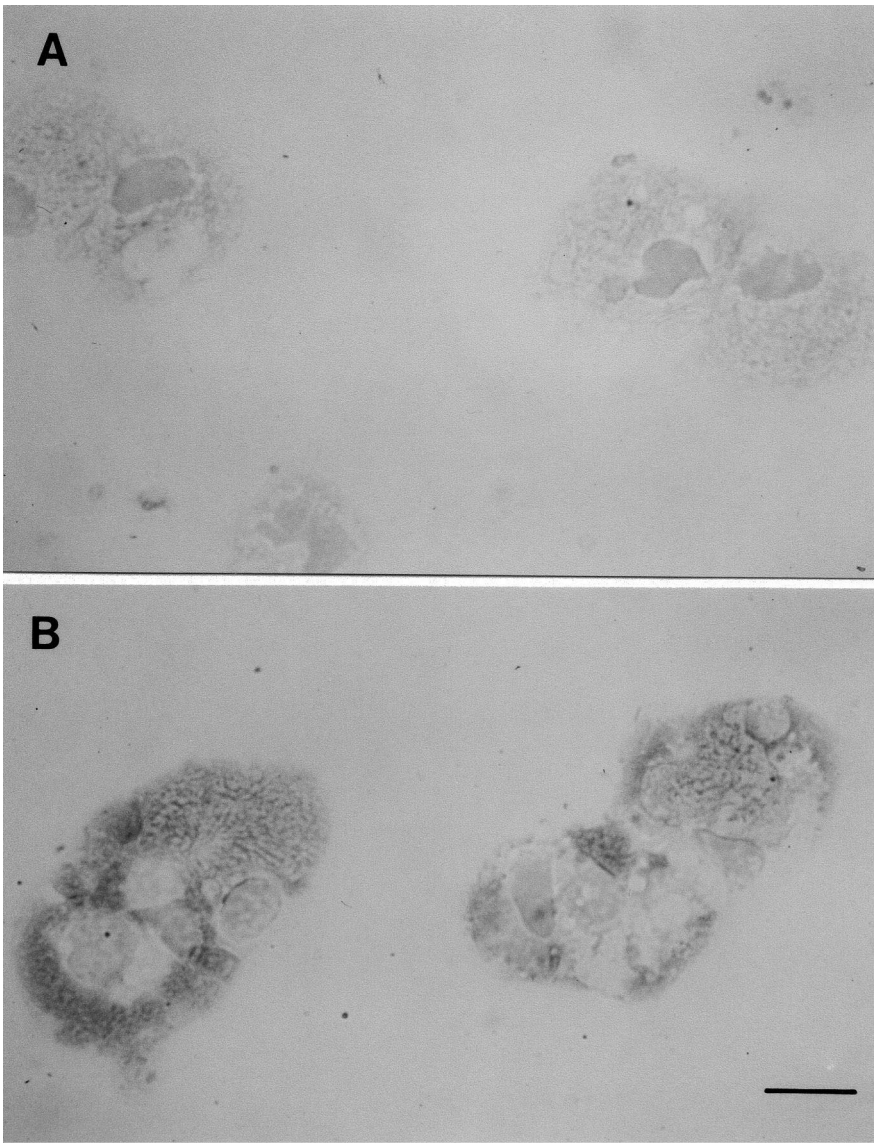

Fig. 1. Immunocytochemistry with an anti-NOS polyclonal antibody using avidin–biotin–peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. The control *Viviparus ater* immunocytes show a weak reaction (A), while increased immunoreactivity is observed in the bacteria-activated cells (B). Bar=10 μm.

pentadecapeptide, FIEESKKDAEVFSS, of rat cerebellar NOS [2] inhibited the molluscan enzyme activity in a concentration-dependent manner [7]. Furthermore, the molluscan NOS enzyme showed values of K_m for L-arginine and K_i for L-NMMA of 2.5 and 4.7 mM, respectively, values comparable to those detected in the enzymes of mammalian cells [7]. Very low free calcium concentrations were able to decrease NOS activity of immunocytes, even when more than 70% of the enzyme activity was still present [7]. This Ca^{2+}-dependence of immunocyte NOS is similar to the inducible form of the NOS cloned from human hepatocytes [21]. Furthermore, NOS genes have been cloned from three insect species, and similarities in the amino acid sequences with those of mammalian Ca^{2+}/calmodulin-dependent NOS's were found [32,52,75].

As far as the biological role of NO in the immune system is concerned, it has been shown that molluscan immunocytes produce a bactericidal substance indirectly identified as NO [17]. Indeed, the immunocytes induced bacterial clumping and killing in a manner similar to that provoked by the NO donor sodium nitroprusside (SNP) [49]. The use of NOS inhibitors, such as L-NMMA and L-NMA, blocked clumping of bacteria around immunocytes. These findings indicate the capability of immunocytes to produce NO, a molecule that, as seen in macrophages, intervenes in defence mechanisms by virtue of its capacity to mediate cytotoxic action [67]. In addition, it has been found that the NO system is not an alternative to phagocytosis, but that both are fundamental in bacterial elimination [49]. In the course of bacterial killing, phagocytosis was activated earlier than bacterial clumping, which occurred later when phagocytosis was still active. NO also acts in insect immunity. Indeed, the increased NO production in *Drosophila melanogaster* and *Drosophila teissieri* observed during immunocyte-mediated melanotic encapsulation of parasitoid eggs suggests that NO may play a role not only as a cytotoxic component, but also as a signaling molecule [44].

NOS in molluscs was induced by different cytokines as demonstrated by in vivo experiments [47,48]. The highest NOS induction was observed 40 min after cytokine stimulation, returning to control levels after 90 min. These data differ from observations in mammalian macrophages, where a single cytokine did not induce NOS [11]. It can be argued that in lower forms of life, ancestral molecules, such as cytokines [46], and processes, such as phagocytosis and the NO system, are correlated, in contrast to higher animals where other factors mediate or intervene in these phenomena.

2. NO and signaling molecules

2.1. NO determination

With regard to the NO studies, a major technological advancement occurred with the development of the NO-selective amperometric probe. This allowed for the detection of NO at low levels and in real time. The probe we have used successfully over the years is one manufactured by World Precision Instruments (Sarasota, FL). Here, the redox current is detected by a current–voltage converter circuit and continuously recorded by the DUO 18 software supplied by World Precision Instruments. The small tip diameter of the probe permitted the use of a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope to position the sensor 3–5 μm above the cell/tissue surface. The system is calibrated daily using different concentrations of a nitrosothiol donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; Sigma, St Louis, MO) to generate a standard curve. The detailed characteristics and selectivity of this probe have been reviewed extensively elsewhere [34]. Furthermore, manipulation/handling of the cells/tissues is performed only with glass instruments, reducing background noise considerably.

2.2. Signaling molecules

2.2.1. Cannabinoid

We have demonstrated that several invertebrates possess elements of the mammalian cannabinoid signaling system, including an endogenous cannabinoid receptor that appears to be type 1 (CBR-1) [72]. Our results to date demonstrate that endocannabinoids (anandamide and 2-arachidonyl glycerol [30]) probably operate via mechanisms similar to those in vertebrates [40,63]. Cannabinoid receptors have been found on both invertebrate neurons and immunocytes [70,72]. These receptors appear to initiate the same cellular events [70,72], i.e. constitutive nitric oxide synthase (cNOS) activation resulting in NO release, and they work via a CBR-1-dependent mechanism [72]. We have also recently shown that NO release occurs in response to receptor activation in mammalian and human tissue [10,74], suggesting that some cannabinoid regulatory processes will be similar in both invertebrates and humans. These experiments demonstrate that this class of compounds is an 'ancient' one that appears to affect many biological activities.

In the mussel and leech, the CBR-1 cannabinoid receptor is present on immunocytes and microglia and its actions are coupled to NO release [70,72] and inhibited by the nitric oxide synthase (NOS) inhibitor *N*-omega-nitro-L-arginine methyl ester (L-NAME; [70,72]). Thus, *Mytilus* and leech neural tissues contain high affinity anandamide receptors that are coupled to NO release [70,72]. It has also been demonstrated that another cannabinoid agonist, CP 55940, can stimulate NO release from *Mytilus* and leech neuronal tissue, whereas SR 141716A, an antagonist, blocks NO release in both invertebrate and human tissues [10,70,72].

We have partially cloned and sequenced the cDNA for the cannabinoid receptor CBR-1 from leech [72]. The sequence is similar to those from human (49%) [57] and

rat (47%) [39]. Strikingly, there are two highly conserved motifs — positions 1–97 and 128–153 — that show 80 and 58% homology to human CBR-1 [72]. Furthermore, leech tissues have been shown to contain endogenous endocannabinoids [38]. The presence of endocannabinoids and their receptor and its coupling to NO release in these organisms, as well as in human tissue, indicates that this signaling system has been conserved during evolution.

2.2.2. Opiate

The ability of invertebrate immunocytes to produce NO has been inferred from earlier studies demonstrating that this compound can initiate cell rounding as well as bactericidal actions [35,49]. Therefore, the ability of invertebrate microglia to produce NO does not come as a complete surprise since this mechanism has also been found in mammals (Fig. 2). Functionally, microglia generate NO that kills cells by inhibition of glycolysis, the TCA cycle and DNA synthesis [33]. As for the association of NO and morphine, this has also been reported in the mammalian literature. NO has been associated with antinociception [13,36] as well as tolerance and dependence [36]. Peripheral morphine analgesia involves a pathway in which NO stimulates cGMP [18]. Morphine depresses concanavalin A-stimulated macrophage NO production [16]. Morphine and NO have been linked to gastrointestinal regulation [25].

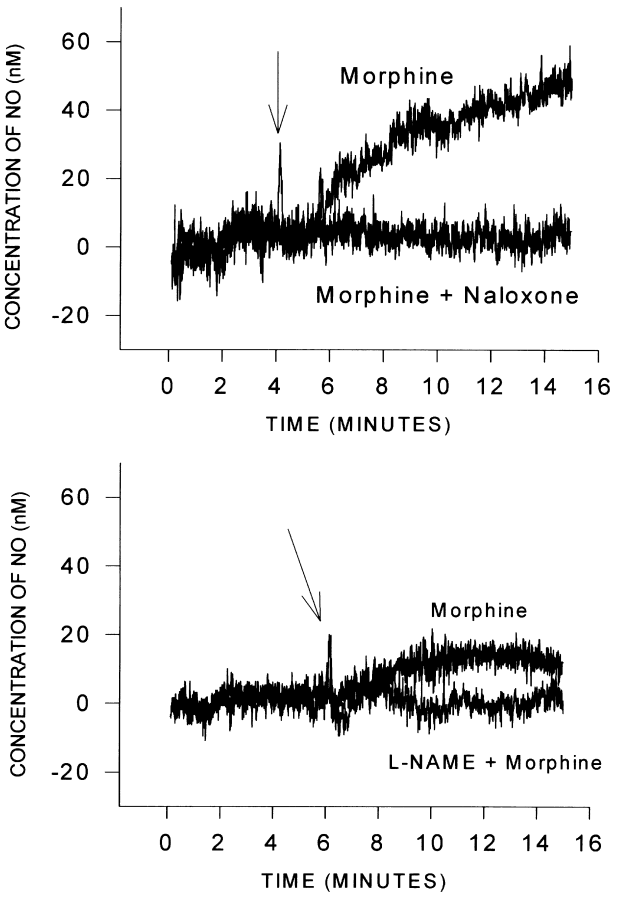

Fig. 2. In vitro stimulation of NO production by morphine (10^{-6} M) and its antagonism by naloxone and L-NAME. Representative recordings of NO in the incubation medium prior to and following opiate exposure with either naloxone (top; 10^{-6} M) or L-NAME (bottom; 10^{-4} M). Arrows denote the time of drug application. For different preparations (top represents microglia from 80 pedal ganglia, the bottom from 40 ganglia) there can be a difference in the magnitude of the response to morphine as noted between the top and bottom curves. In this representative set of experiments, the top panel value for NO produced at 10 min was 39 nM, whereas the bottom was 18 nM compared to a calibration curve for SNAP. A 25-μm tip probe was inserted into the medium and placed about 10 μm above the cells on an inverted microscope stage (Nikon Diaphot).

2.2.2.1. Microglia and NO. The results of an earlier study add considerable weight to the concept that in invertebrates a distinct subset of glial cells is closely related to immunoactive hemocytes (immunocytes) and, therefore, may be judged to correspond to vertebrate microglia [58]. The existence of more than one type of invertebrate glial cell had been proposed as early as 1883 by Vignal and subsequently by a number of investigators [58]. However, in these studies the term ‘microglia’ was used only very rarely and primarily on the basis of morphological criteria comparable to those known in vertebrate microglia. The evidence in support of this claim is twofold: (a) In response to surgical trauma, a subset of reactive glial cells leaving the invertebrate ganglia exhibit a number of features characteristic of the animals’ immunocytes; (b) These structural and functional commonalities parallel those between microglia and macrophages observed in vertebrates under comparable experimental conditions [53]. The pertinent points can be summarized as follows: (1) In both groups of animals comparable mobile immune cells are known to enter various tissues, including nervous tissue; (2) In the process of transformation to resident glia, these immunocytes appear to undergo similar conformational changes and some biochemical dedifferentiation; (3) During their egress from lesioned neural tissue, in excised ganglia maintained in incubation medium, the glial ‘sentinel cells’ regain some of their inherent characteristics, including an ameboid conformation, adhesiveness, and motility; (4) An important criterion for the immunoregulatory potential of these mobilized microglial cells and their counterpart in vertebrates is their phagocytotic activity. This capacity is made use of in the uptake and removal of degenerating neural structures; (5) In both groups of animals, the egress of reactivated microglial cells from lesioned nervous tissue and their conformational changes are counteracted by exogenous morphine, a process corresponding to the inhibiting effect of this drug in immune cells [64]; (6) An additional point to be made is that a given immunoregulatory molecule may be either present in both cell types under consideration here or else absent in both. For example, cytokines (IL-1 in particular) have been demonstrated in microglial and immunoreactive vertebrate cells [19] and likewise in immunocytes as well as microglial cells of an invertebrate, *Mytilus edulis* [50].

Morphine has been shown to be an endogenous signaling molecule in *Mytilus* neural and immune tissues

[23,24,64,68,69,73,76–78]. Exposure of excised *Mytilus* pedal ganglia to morphine for 24 h resulted in a significant dose-dependent decrease in microglial egress that was naloxone-sensitive [58]. In co-incubating the excised ganglia with morphine and the NOS inhibitor, *N*-omega-nitro-L-arginine methyl ester (L-NAME), an increase in microglial egress was observed, suggesting that morphine may stimulate microglia to release NO [31]. Morphine exposure to these cells in vitro resulted in NO release (39.4±4.9 nM), a phenomenon found to be naloxone-sensitive (10^{-6} M; NO level=5.9±2.6 nM) and L-NAME-sensitive (10^{-4} M; NO level=2.8±1.8 nM) (Fig. 2) [31]. Opioid peptides did not stimulate NO release, indicating that the process was mediated by the opiate alkaloid selective μ_3 receptor. Co-incubation of microglia with L-arginine or the superoxide scavenger, superoxide dismutase, resulted in the observation of significantly higher NO levels following morphine stimulation [31]. Taken together, the data demonstrate that morphine can stimulate NO release in cells obtained from an invertebrate, an animal 500 million years divergent in evolution from man. These observations underscore the significance of this process and further substantiate the critical importance of morphine as a naturally occurring signaling molecule.

Supporting the above observations are studies demonstrating that morphine inhibits the movement of mammalian and invertebrate immunocytes and microglia from excised vertebrate and invertebrate ganglia [59,62,74].

2.2.2.2. Neural NO. In a 1997 study, we demonstrated that *Mytilus* pedal ganglia can respond to both morphine and anandamide exposure by releasing cNOS-derived NO [71]. This effect occurred via antagonism by the respective receptors' inhibitors as well as by L-NAME sensitivity. Furthermore, we linked the release of NO to an action inhibiting ganglionic dopamine release but not serotonin, demonstrating specificity.

Studies are emerging that document the involvement of NO in various physiological functions of invertebrates, such as olfaction [15,22] and epithelial function [12]. Furthermore, NOS immunoreactivity has been found in various invertebrate tissues [3,8,29,37,43,51]. The application of NO via NO-donors has also been demonstrated to have intracellular actions [1] as well as to influence immunocyte behavior [49].

2.3. Vascular NO

It is widely accepted that invertebrates have an open circulatory system as opposed to the closed tubular defined system found in vertebrates. Despite this anatomical difference, mollusks do contain a pulsating heart tissue, in addition to, as noted above, immunocytes. In many reports regarding vascular tissues, NO has been shown to inhibit white blood cell adherence [62]. Thus, it was of interest to determine if this same phenomenon would occur in the respective *Mytilus* tissues. We demonstrate that *Mytilus* immunocytes have the ability to adhere to the organisms' heart tissue (Fig. 3) and that acetylcholine (ACH) has the ability to significantly diminish this adherence when exposed to heart tissue (Fig. 3). Further, ACH performs this action by stimulating cNOS-derived NO release (Fig. 4). The addition of L-NAME (10^{-4} M) blocks the ACH effect on immunocyte adherence by 87%, substantiating the involvement of NO. Thus, we surmise that basal NO levels originating from the organisms' heart tissue serve to limit cellular adherence, as is the case in mammals. Clearly, this action is important because it keeps the immunocytes in a mobile state ready to descend on a proinflammatory event in both types of animals.

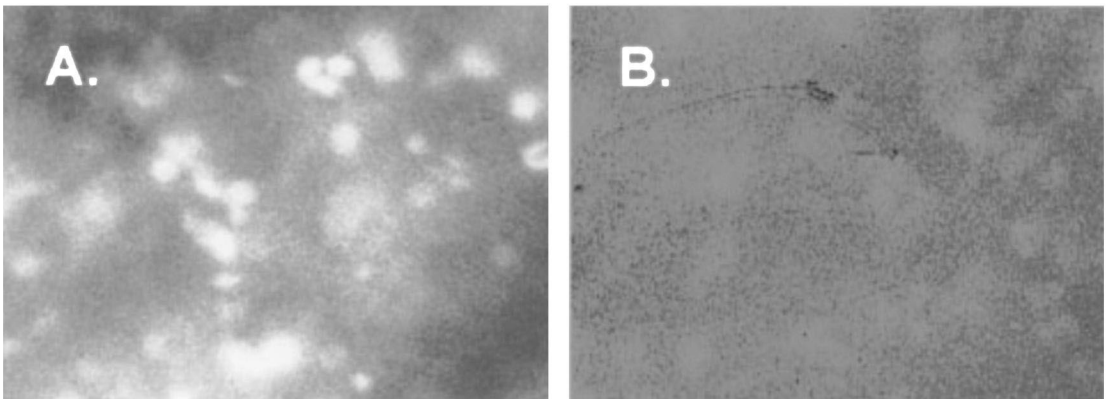

Fig. 3. After aspiration from the heart cavity, 10^{-6} M immunocytes were centrifuged at 8000 rpm for 5 min. The supernatant was discarded, and the cells treated with the fluorescent vital dye, PKH26-GL (Sigma, St Louis, MO) in accordance with the detailed instructions of the commercially available kit. Briefly, cells were treated with the fluorescent dye solution for 2 min with gentle agitation followed by addition of 2 ml of hemolymph to stop the reaction. The cells were then washed extensively with phosphate buffered saline (PBS) to remove unincorporated dye, and subsequently diluted and divided into 100-μl volumes which contained approximately 412±15 cells (see Ref. [5] for details). Panel A represents cells adhering on the surface of the heart tissue whereas panel B represents heart tissue exposed to 10^{-8} M ACH, stimulating NO and inhibiting cellular adherence.

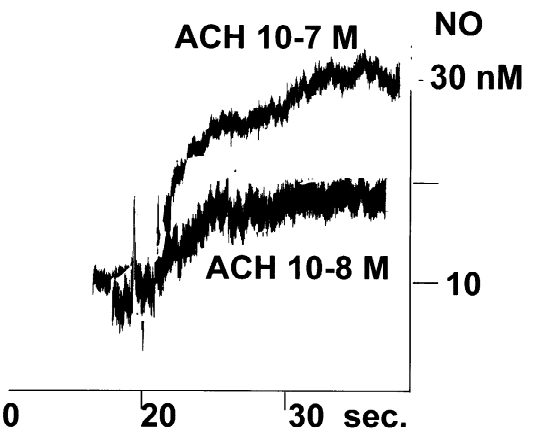

Fig. 4. In vitro stimulation of NO production by acetylcholine (ACH) in *Mytilus* heart tissue. Representative recordings of NO in the incubation medium prior to and following ACH exposure at the 20-s mark. In this representative set of experiments, the top panel value for NO produced at 1 min was 33 nM, whereas the bottom was 18 nM compared to a calibration curve for SNAP. A 25-μm tip probe was inserted into the medium and placed about 10 μm above the tissue on an inverted microscope stage (Nikon Diaphot).

3. Conclusions

With regard to the possible organismic significance of these observations, we can only surmise that given any immune-activating stimulus, naturally occurring morphine has the potential to eventually down regulate this excitatory process [68,73,74]. However, given the excessive trauma of ganglionic excision, this immune down regulating mechanism may simply not work, yet it will make itself evident [68,73,74]. As to the question of why morphine and cannabinoid are linked to NO regulation, the answer may be found in NO's intracellular regulatory activities, including DNA modulation. This mechanism may also explain how the small amount of NO release for such a short period of time may be so potent, as noted by the inhibition of microglial egress for 24 h.

Nitric oxide is a major signaling molecule in the immune, cardiovascular and nervous systems of mammals [67]. We note in the present data that this same molecule is present and performs similar functions in the invertebrate counterpart of these physiological systems. In mammals, the synthesizing enzyme, NOS, occurs in three forms: endothelial (e), neuronal (n) and inducible (i) NOS. It appears that these similarities exist in invertebrates as well since NO can be produced both rapidly and after a latent period. The first two enzymes are constitutively expressed. We surmise that in many tissues there is a basal level of NO and that the actions of several signaling molecules initiate increases in cNOS-derived NO to enhance momentary basal levels that then exert inhibitory cellular actions, via cellular conformational changes [67]. It is our contention that much of the literature concerning the actions of NO really measures i-NOS-derived NO activity. We make the case that cNOS is responsible for a basal or 'tonal' level of NO; that this NO keeps particular types of cells in a state of inhibition and that activation of these cells occurs through disinhibition [67]. Furthermore, naturally occurring signaling molecules such as morphine and anandamide appear to exert in part, their beneficial physiological actions (such as immune and endothelial down regulation) by the stimulation of cNOS. With regard to opiates, we demonstrate the presence of a human endothelial mu opiate receptor by RT-PCR and sequence determination, further substantiating the role of opiates in vascular coupling to NO release as is the case for invertebrates [6]. Taken together, cNOS-derived NO enhances basal NO actions, i.e. cellular activation state, and these actions are further enhanced by iNOS-derived NO.

In summary, it appears NO processes evolved first in invertebrates and were retained during evolution. Thus, NO's biochemical modulatory processes were first developed in these non-cognitive organisms. In man, these basal biochemical NO-mediated processes are used in cognitive processes, i.e. the relaxation response [65,66]. It would appear, therefore, that during evolution the biochemical NO-mediated processes were retained and new functions added to accommodate cognition.

Acknowledgements

The work was in part supported by the following grants NIDA 09010, MH-DA COR 17138.

References

[1] V. Adam-Vizi, T.J. Allen, P.F. Baker, The effects of nitroprusside and putative agonists on guanylate cyclase activity in squid giant axons, Biochim. Biophys. Acta 938 (1988) 461–468.
[2] P. Alm, B. Larsson, E. Ekblad, F. Sundler, K.E. Andersson, Immunohistochemical localization of peripheral nitric oxide synthase-containing nerves using antibodies raised against synthesized C- and N-terminal fragments of a cloned enzyme from rat brain, Acta Physiol. Scand. 148 (1993) 421–429.
[3] A. Barnhart, E. Orona, Nitric oxide synthase staining in lobster olfactory system, Chem. Senses 19 (1994) 438–442.
[4] G. Bicker, NO news from insect brains, Trends Neurosci. 21 (1998) 349–355.
[5] T.V. Bilfinger, A. Hartman, Y. Liu, H.I. Magazine, G.B. Stefano, Cryopreserved veins used for myocardial revascularization: a 5 year experience and a possible mechanism for their increased failure, Ann. Thorac. Surg. 63 (1997) 1063–1069.
[6] P. Cadet, G.B. Stefano, *Mytilus edulis* pedal ganglia express μ opiate receptor transcripts exhibiting high sequence identity with human neuronal μ1, Mol. Brain Res. 74 (1999) 242–246.
[7] A. Conte, E. Ottaviani, Nitric oxide synthase activity in molluscan hemocytes, FEBS Lett. 365 (1995) 120–124.
[8] I.R. Cooke, S.L. Edwards, C.R. Anderson, The distribution of NADPH diaphorase activity and immunoreactivity to nitric oxide synthase in the nervous system of the pulmonate mollusc *Helix aspersa*, Cell Tissue Res. 277 (1994) 565–572.
[9] T.M. Dawson, D.S. Bredt, M. Fotuhi, P.M. Hwang, S.H. Snyder, Nitric oxide synthase and neuronal NADPH diaphorase are identical

in brain and peripheral tissues, Proc. Natl. Acad. Sci. USA 88 (1991) 7797–7801.
[10] D.G. Deutsch, M.S. Goligorsky, P.C. Schmid, R.J. Krebsbach, H.H.O. Schmid, S.K. Das, S.K. Dey, G.B. Stefano, R. Arreaza, C. Thorup, L. Moore, Production and physiological actions of anandamide in the vasculature of the rat kidney, J. Clin. Invest. 100 (1997) 1538–1546.
[11] A.H. Ding, C.F. Nathan, D.J. Stuehr, Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production, J. Immunol. 141 (1988) 2407–2412.
[12] J.A. Dow, S.H. Maddrell, S.A. Davies, N.J. Skaer, K. Kaiser, A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*, Am. J. Physiol. 266 (1994) R1716–R1719.
[13] I.D. Duarte, S.H. Ferreira, The molecular mechanism of central analgesia induced by morphine or carbachol and the L-arginine–nitric oxide–cGMP pathway, Eur. J. Pharmacol. 221 (1992) 171.
[14] R. Elofsson, M. Carlberg, L. Moroz, L. Nezlin, D. Sakharov, Is nitric oxide (NO) produced by invertebrate neurones?, Neuroreport 4 (1993) 279–282.
[15] M.R. Elphick, G. Kemenes, K. Staras, M. O'Shea, Nitric oxide mediates chemosensory activation of feeding in *Lymnea stagnalis*, Soc. Neurosci. Abstr. 20 (1994) 1281–1285.
[16] K. Fecho, K.A. Maslonek, M.E. Coussons-Read, L.A. Dykstra, D.T. Lysle, Macrophage-derived nitric oxide is involved in the depressed concanavalin A responsiveness of splenic lymphocytes from rats administered morphine in vivo, J. Immunol. 152 (1994) 5845–5851.
[17] M. Feelisch, E. Noack, The in vitro metabolism of nitrovasodilators and their conversion into vasospective species, in: B.S. Lewis, A. Kimch (Eds.), Heart Failure — Mechanisms and Management, Springer, Berlin, 1991, pp. 241–255.
[18] S.H. Ferreira, I.D. Duarte, B.B. Lorenzetti, The molecular mechanism of action of peripheral morphine analgesia: stimulation of the cGMP system via nitric oxide release, Eur. J. Pharmacol. 201 (1991) 121.
[19] A. Fontana, F. Kristensen, R. Dubs, D. Gemsa, E. Weber, Production of prostaglandin E and interleukin-1 like factors by cultured astrocytes and c-6 glioma cells, J. Immunol. 129 (1982) 2413–2419.
[20] A. Franchini, P. Fontanili, E. Ottaviani, Invertebrate immunocytes: relationship between phagocytosis and nitric oxide production, Comp. Biochem. Physiol. 110B (1995) 403–407.
[21] D.A. Geller, C.J. Lowenstein, R.A. Shapiro, A.K. Nussler, M. Di Silvio, S.C. Wang, D.K. Nakayama, R.L. Simmons, S.H. Snyder, T.R. Billiar, Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes, Proc. Natl. Acad. Sci. USA 90 (1993) 3491–3495.
[22] A. Gelperin, Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusc, Nature 369 (1994) 61–63.
[23] Y. Goumon, F. Casares, S. Pryor, L. Ferguson, B. Brownwell, P. Cadet, C.M. Rialas, I. Welters, D. Sonetti, G.B. Stefano, *Ascaris suum*, an internal parasite, produces morphine, J. Immunol. 165 (2000) 339–343.
[24] Y. Goumon, F. Casares, W. Zhu, G.B. Stefano, The presence of morphine in ganglioic tissues of *Modiolus deminissus*: A highly sensitive method of quantitation for morphine and its derivatives, Mol. Brain Res. 86 (2001) 184–188.
[25] K. Gyires, The role of endogenous nitric oxide in the gastroprotective action of morphine, Eur. J. Pharmacol. 255 (1994) 33–37.
[26] B.T. Hope, G.J. Michael, K.M. Knigge, S.R. Vincent, Neuronal NADPH diaphorase is a nitric oxide synthase, Proc. Natl. Acad. Sci. USA 88 (1991) 2811–2814.
[27] S. Huang, H.H. Kerschbaum, A. Hermann, Nitric oxide-mediated cGMP synthesis in *Helix* neural ganglia, Brain Res. 780 (1998) 329–336.
[28] J.W. Jacklet, M. Gruhn, Co-localization of NADPH-diaphorase and myomodulin in synaptic glomeruli of *Aplysia*, Neuroreport 5 (1994) 1841–1844.
[29] K.U. Johansson, M. Carlberg, NADPH-diaphorase histochemistry and nitric oxide synthase activity in deutocerebrum of the crayfish, *Pacifastacus leniusculus* (Crustacea, Decapoda), Brain Res. 649 (1994) 36–42.
[30] J. Lai, E.J. Bilsky, R.B. Rothman, F. Porreca, Treatment with antisense oligonucleotides in the opioid receptor selectively inhibits 2-agonist antinociception, Neuroreport 5 (1994) 1049.
[31] Y. Liu, D. Shenouda, T.V. Bilfinger, M.L. Stefano, H.I. Magazine, G.B. Stefano, Morphine stimulates nitric oxide release from invertebrate microglia, Brain Res. 722 (1996) 125–131.
[32] S. Luckhart, Y. Vodovotz, L. Cui, R. Rosenberg, The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide, Proc. Natl. Acad. Sci. USA 95 (1998) 5700–5705.
[33] H.I. Magazine, Current trends and future directions, Adv. Neuroimmunol. 5 (1995) 479–490.
[34] H.I. Magazine, Detection of endothelial cell-derived nitric oxide: current trends and future directions, Adv. Neuroimmunol. 5 (1995) 479–485.
[35] H.I. Magazine, Y. Liu, T.V. Bilfinger, G.L. Fricchione, G.B. Stefano, Morphine-induced conformational changes in human monocytes, granulocytes, and endothelial cells and in invertebrate immunocytes and microglia are mediated by nitric oxide, J. Immunol. 156 (1996) 4845–4850.
[36] N.H. Majeed, B. Przewlocka, H. Machelska, R. Przewlocki, Inhibition of nitric oxide synthase attenuates the development of morphine tolerance and dependence in mice, Neuropharmacology 33 (1994) 189.
[37] A. Martinez, V. Riveros-Moreno, J.M. Polak, S. Moncada, P. Sesma, Nitric oxide (NO) synthase immunoreactivity in the starfish *Marthasterias glacialis*, Cell Tissue Res. 275 (1994) 599–603.
[38] I. Matias, T. Bisogno, D. Melck, F. Vandenbulcke, M. Verger-Bocquet, L. De Petrocellis, C. Sergheraert, C. Breton, V. Di Marzo, M. Salzet, Evidence for an endocannabinoid system in the central nervous system of the leech *Hirudo medicinalis*, Brain Res. Mol. Brain Res. 87 (2001) 145–159.
[39] L.A. Matsuda, S.J. Lolait, M.J. Brownstein, A.C. Young, T.I. Bonner, Structure of a cannabinoid receptor and functional expression of the cloned cDNA, Nature 346 (1990) 561–564.
[40] D.W. Mattocks, M. Salzet, B. Salzet, G.B. Stefano, Anandamide-induced conformational changes in leech and mussel immunocytes are mediated by nitric oxide, Anim. Biol. 6 (1997) 73–77.
[41] S. Moncada, R.M.J. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43 (1991) 109–142.
[42] L.L. Moroz, J.H. Park, W. Winlow, Nitric oxide activates buccal motor patterns in *Lymnaea stagnalis*, Neuroreport 4 (1993) 643–646.
[43] L.L. Moroz, A.J. Roylance, Antibodies against cerebellar nitric oxide (NO) synthase label cells in the CNS and peripheral tissues of the freshwater snail *Lymnea stagnalis*, J. Physiol. 473 (1993) 28–32.
[44] A.J. Nappi, E. Vass, F. Frey, Y. Carton, Nitric oxide involvement in *Drosophila* immunity, Nitric Oxide 4 (2000) 423–430.
[45] A. Nighorn, N.J. Gibson, D.M. Rivers, J.G. Hildebrand, D.B. Morton, The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*, J. Neurosci. 18 (1998) 7244–7255.
[46] E. Ottaviani, C. Franceschi, The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems, Immunol. Today 18 (1997) 169–174.
[47] E. Ottaviani, A. Franchini, S. Cassanelli, S. Genedani, Cytokines and invertebrate immune responses, Biol. Cell 85 (1995) 87–91.
[48] E. Ottaviani, A. Franchini, D. Sonetti, G.B. Stefano, Antagonizing effect of morphine on the mobility and phagocytic activity of invertebrate immunocytes, Eur. J. Pharmacol. 276 (1995) 35–39.

[49] E. Ottaviani, L.R. Paemen, P. Cadet, G.B. Stefano, Evidence for nitric oxide production and utilization as a bacteriocidal agent by invertebrate immunocytes, Eur. J. Pharmacol. 248 (1993) 319–324.
[50] L.R. Paemen, E. Porchet-Hennere, M. Masson, M.K. Leung, T.K. Hughes, G.B. Stefano, Glial localization of interleukin-1a in invertebrate ganglia, Cell. Mol. Neurobiol. 12 (1992) 463–472.
[51] M.W. Radomski, J.F. Martin, S. Moncada, Synthesis of nitric oxide by the haemocytes of the American horseshoe crab *(Limulus polyphemus)*, Phil. Trans. R. Soc. Lond. B Biol. Sci. 334 (1991) 129–133.
[52] M. Regulski, T. Tully, Molecular and biochemical characterization of dNOS: a Drosophila Ca^{2+}/calmodulin-dependent nitric oxide synthase, Proc. Natl. Acad. Sci. USA 92 (1995) 9072–9076.
[53] E. Rieske, M.B. Graeber, W. Tetzlaff, A. Czlonkowska, W.J. Streit, G.W. Kreutzberg, Microglia and microglia-derived brain macrophages in culture: generation from axotomized rat facial nuclei, identification and characterization in vitro, Brain Res. 492 (1989) 1–14.
[54] M. Salzet, C. Cocquerelle, M. Verger-Bocquet, S.C. Pryor, C.M. Rialas, V. Laurent, G.B. Stefano, Leech immunocytes contain proopiomelanocortin: nitric oxide mediates hemolymph POMC processing, J. Immunol. 159 (1997) 5400–5411.
[55] M. Salzet, G.B. Stefano, A renin-like enzyme in the leech *Theromyzon tessulatum*, Mol. Cell. Endocrinol. 131 (1997) 1–8.
[56] M. Salzet, G.B. Stefano, Invertebrate proenkephalin: Delta opioid binding sites in leech ganglia and immunocytes, Brain Res. 768 (1997) 232.
[57] D. Shire, C. Carillon, M. Khaghad, B. Calendra, M. Rinaldi-Carmona, G. Le Fur, D. Caput, P. Ferrara, An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing, J. Biol. Chem. 270 (1995) 3726–3731.
[58] D. Sonetti, E. Ottaviani, F. Bianchi, M. Rodriquez, M.L. Stefano, B. Scharrer, G.B. Stefano, Microglia in invertebrate ganglia, Proc. Natl. Acad. Sci. USA 91 (1994) 9180–9184.
[59] D. Sonetti, E. Ottaviani, G.B. Stefano, Opiate signaling regulates microglia activities in the invertebrate nervous system, Gen. Pharmacol. 29 (1997) 39–47.
[60] G.B. Stefano, Comparative aspects of opioid–dopamine interaction, Cell. Mol. Neurobiol. 2 (1982) 167–178.
[61] G.B. Stefano, Role of opioid neuropeptides in immunoregulation, Prog. Neurobiol. 33 (1989) 149–159.
[62] G.B. Stefano, Autoimmunovascular regulation: morphine and anandamide stimulated nitric oxide release, J. Neuroimmunol. 83 (1998) 70–76.
[63] G.B. Stefano, T.V. Bilfinger, C.M. Rialas, D.G. Deutsch, Arachidonyl-glycerol stimulates nitric oxide from human immune and vascular tissues and invertebrate immunocytes by cannabinoid receptor 1, Pharmacol. Res. 42 (2000) 317–322.
[64] G.B. Stefano, A. Digenis, S. Spector, M.K. Leung, T.V. Bilfinger, M.H. Makman, B. Scharrer, N.N. Abumrad, Opiatelike substances in an invertebrate, a novel opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression, Proc. Natl. Acad. Sci. USA 90 (1993) 11099–11103.
[65] G.B. Stefano, G.L. Fricchione, B.T. Slingsby, H. Benson, The placebo effect and relaxation response: Neural processes and their coupling to constitutive nitric oxide, Brain Res. Brain Res. Rev. 35 (2000) 1–19.
[66] G.B. Stefano, Z.I. Fulop, C.M. Rialas, P. Cadet, W. Zhu, M. Rotondo, P. Ghuman, G. Koutsovasilis, S. Spalluto, M. Smith, G.L. Fricchione, H. Benson. Nitric oxide is involved in the relaxation response, Brain Res. Cogn. Brain Res. (2001) in press.
[67] G.B. Stefano, Y. Goumon, T.V. Bilfinger, I. Welters, P. Cadet, Basal nitric oxide limits immune, nervous and cardiovascular excitation: Human endothelia express a mu opiate receptor, Prog. Neurobiol. 60 (2000) 531–544.
[68] G.B. Stefano, Y. Goumon, F. Casares, P. Cadet, G.L. Fricchione, C. Rialas, D. Peter, D. Sonetti, M. Guarna, I. Welters, E. Bianchi, Endogenous morphine, Trends Neurosci. 9 (2000) 436–442.
[69] G.B. Stefano, M.K. Leung, T.V. Bilfinger, B. Scharrer, Effect of prolonged exposure to morphine on responsiveness of human and invertebrate immunocytes to stimulatory molecules, J. Neuroimmunol. 63 (1995) 175–181.
[70] G.B. Stefano, Y. Liu, M.S. Goligorsky, Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia, and human monocytes, J. Biol. Chem. 271 (1996) 19238–19242.
[71] G.B. Stefano, B. Salzet, C.M. Rialas, M. Pope, A. Kustka, K. Neenan, S.C. Pryor, M. Salzet, Morphine and anandamide stimulated nitric oxide production inhibits presynaptic dopamine release, Brain Res. 763 (1997) 63–68.
[72] G.B. Stefano, B. Salzet, M. Salzet, Identification and characterization of the leech CNS cannabinoid receptor: Coupling to nitric oxide release, Brain Res. 753 (1997) 219–224.
[73] G.B. Stefano, B. Scharrer, Endogenous morphine and related opiates, a new class of chemical messengers, Adv. Neuroimmunol. 4 (1994) 57–68.
[74] G.B. Stefano, B. Scharrer, E.M. Smith, T.K. Hughes, H.I. Magazine, T.V. Bilfinger, A. Hartman, G.L. Fricchione, Y. Liu, M.H. Makman, Opioid and opiate immunoregulatory processes, Crit. Rev. Immunol. 16 (1996) 109–144.
[75] M. Yuda, M. Hirai, K. Miura, H. Matsumura, K. Ando, Y. Chinzei, cDNA cloning, expression and characterization of nitric-oxide synthase from the salivary glands of the blood-sucking insect *Rhodnius prolixus*, Eur. J. Biochem. 242 (1996) 807–812.
[76] W. Zhu, G. Baggerman, Y. Goumon, F. Casares, B. Brownawell, G.B. Stefano, Presence of morphine and morphine-6-glucuronide in the marine mollusk *Mytilus edulis* ganglia determined by GC/MS and Q-TOF-MS. Starvation increases opiate alkaloid levels, Brain Res. Mol. Brain Res. 88 (2001) 155–160.
[77] W. Zhu, G. Baggerman, Y. Goumon, M.H. Zenk, G.B. Stefano, Identification of morphine and morphine-6-glucuronide in the adrenal medullary chromaffin PC-12 cell line by nano electrospray ionization double quadrupole orthogonal acceleration time of flight mass spectrometry, Eur. J. Mass Spectrom. 7 (2001) 25–28.
[78] W. Zhu, Y. Goumon, F. Casares, B. Brownwell, D. Sonetti, G.B. Stefano, The presence of morphine and morphine 6 glucuronide in *Mytilus edulis* ganglia, Mol. Brain Res. (2001) in press.