The Journal of Experimental Biology 207, 2845-2857
Published by The Company of Biologists 2004
doi:10.1242/jeb.01117

A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis*

Hyun-Woo Kim, Luisa A. Batista, Jodi L. Hoppes, Kara J. Lee and Donald L. Mykles*

Department of Biology, Colorado State University, Fort Collins, CO 80523, USA

*Author for correspondence (e-mail: don@lamar.colostate.edu)

Accepted 27 May 2004

Summary

NO signaling is involved in many physiological processes in invertebrates. In crustaceans, it plays a role in the regulation of the nervous system and muscle contraction. Nested reverse transcription-polymerase chain reaction (RT-PCR) and 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR generated a full-length cDNA sequence (3982 bp) of land crab NO synthase (Gl-NOS) from molting gland (Y-organ) and thoracic ganglion mRNA. The open reading frame encoded a protein of 1199 amino acids with an estimated mass of 135 624 Da. Gl-NOS had the highest sequence identity with insect NOS. The amino acid sequences for binding heme and tetrahydrobiopterin in the oxygenase domain, binding calmodulin and binding FMN, FAD and NADPH in the reductase domain were highly conserved. Gl-NOS had single amino acid differences in all three highly conserved FAD-binding sequences, which distinguished it from other NOS sequences. RT-PCR showed that the Gl-NOS mRNA was present in testis, ovary, gill, eyestalk neural ganglia, thoracic ganglion and Y-organ. NOS mRNA varied between preparations of Y-organ, thoracic ganglion and gill, while NOS mRNA was at consistently high levels in the ovary, testis and eyestalk ganglia. Immunohistochemistry confirmed that the Gl-NOS protein was expressed in Y-organ, ovary and gill. These results suggest that NOS has functions in addition to neuromodulation in adults, such as regulating or modulating ecdysteroid synthesis in the Y-organ.

Key words: Crustacea, Arthropoda, nitric oxide synthase, Y-organ, gonad, ovary, gill, eyestalk ganglion, molting gland, nervous tissue, tissue distribution, cDNA cloning, DNA sequence, amino acid sequence, gene expression, mRNA.

Introduction

Nitric oxide (NO) appears to have evolved as a signaling molecule before the radiation of the metazoans (Feelisch and Martin, 1995; Torreilles, 2001). NO is generated by nitric oxide synthase (NOS) from L-arginine, O_2 and NADPH and diffuses freely across the cell membrane to induce responses in neighboring cells (Colasanti and Venturini, 2000). The best-known NO signaling pathway is one in which NO activates a soluble class I guanylyl cyclase (GC-I; Baranano and Snyder, 2001). Activated GC-I produces cyclic 3′,5′-guanosine monophosphate (cGMP), which in turn activates cGMP-dependent protein kinase. In mammals, NO/cGMP signaling is involved in vasodilation, neurotransmission and the immune response (Ahern et al., 2002; Baranano and Snyder, 2001; Bredt and Snyder, 1994). In insects, NO signaling is involved in many physiological processes (Davies, 2000). NO regulates nervous system development and integration (Bicker, 2001; Haase and Bicker, 2003; Schachtner et al., 1999; Seidel and Bicker, 2002; Truman et al., 1996). The hematophagous insect *Rhodnius prolixus* produces NO, which dilates blood vessels and inhibits platelet aggregation in the host (Ribeiro and Nussenzveig, 1993). Recent studies show that the NO/cGMP pathway is involved in the insect immune response (Imamura et al., 2002; Luckhart et al., 1998; Weiske and Wiesner, 1999) and activation of NO/cGMP signaling inhibits steroid synthesis in the ovary of blow fly (*Phormia regina*; Maniere et al., 2003).

In mammals, there are three *NOS* genes: neuronal NOS (*nNOS*), endothelial NOS (*eNOS*) and inducible NOS (*iNOS*) (Bogdan, 2001; Mungrue et al., 2003; Nathan and Xie, 1994). Although their expression and biological roles vary, they share a common structural organization (Ghosh and Salerno, 2003; Kone et al., 2003; Torreilles, 2001). The native enzyme is a homodimer of 130–160-kDa subunits (Torreilles, 2001). The N-terminal oxygenase domain contains the binding motif for a P450-like cysteine thiolate-ligate heme and tetrahydrobiopterin (H4). The C-terminal reductase domain contains the binding motifs for FAD, FMN and NADPH. These two domains are linked by a calmodulin (CaM) binding motif. nNOS and eNOS are constitutively expressed and their enzymatic activities are regulated by the intracellular Ca^{2+} concentration through binding of Ca^{2+} to CaM (Roman et al., 2002). They contain a 40–50 amino acid sequence linked to the FMN binding motif that acts as an autoinhibitory loop, blocking electron transfer from FMN to the heme in the absence of Ca^{2+}/CaM (Craig et al., 2002; Ghosh and Salerno,

2003; Nishida and de Montellano, 2001; Salerno et al., 1997). By contrast, iNOS lacks the autoinhibitory loop and binds CaM with high affinity at low Ca^{2+} levels; its activity is regulated predominantly at the transcriptional level (Chen and Wu, 2003; Nathan and Xie, 1994).

Insect NOSs have the highest sequence identity with mammalian nNOS and share the same organization in the oxygenase, CaM-binding and reductase domains (Davies, 2000; Torreilles, 2001). Insect NOS requires NADPH, Ca^{2+} and CaM for enzymatic activity. It is expressed in a variety of adult and embryonic tissues, including abdominal nerve cord, optic lobes, fat body, antenna, hemocytes, midgut and Malpighian tubule (Broderick et al., 2003; Gibbs and Truman, 1998; Imamura et al., 2002; Luckhart et al., 1998; Nighorn et al., 1998). Isoforms of NOS are generated by alternative splicing. The *Drosophila NOS* gene contains at least four alternative promoters (Stasiv et al., 2001). Some truncated alternative splicing variants of the *Drosophila* NOS lacking the reductase domain may act as dominant negative regulators, as heterodimers would lack enzyme activity (Stasiv et al., 2001).

In crustaceans, NO/cGMP signaling plays a role in neuronal development and neuron, skeletal muscle and cardiac muscle regulation (Aonuma et al., 2000; Aonuma and Newland, 2001, 2002; Erxleben and Hermann, 2001; Hermann and Erxleben, 2001; Johansson and Mellon, 1998; Mahadevan et al., 2004; Scholz, 1999, 2001; Scholz et al., 1998, 2001). NOS is expressed in neurons of the cerebral, stomatogastric, eyestalk, abdominal terminal and cardiac ganglia (Christie et al., 2003; Johansson and Carlberg, 1994; Johansson and Mellon, 1998; Lee et al., 2000; Scholz et al., 2002; Schuppe et al., 2001a,b, 2002; Talavera et al., 1995; Zou et al., 2002). The NO/cGMP signaling pathway is required for the dynamic assembly of the neuronal circuit that drives rhythmic movement in crabs (Scholz, 2001; Scholz et al., 2002), alters ion channel properties of skeletal muscle (Erxleben and Hermann, 2001; Hermann and Erxleben, 2001) and decreases heartbeat amplitude and frequency (Mahadevan et al., 2004). The biochemical properties of crustacean NOS are similar to those of mammalian nNOS and insect NOS, as it also requires NADPH, Ca^{2+} and CaM for activity (Johansson and Carlberg, 1994; Lee et al., 2000; Scholz et al., 2002; Zou et al., 2002).

The NO/cGMP signaling pathway may be involved in regulating molting in crustaceans. Molt inhibiting hormone (MIH), a neuropeptide synthesized in a neurosecretory center (X-organ/sinus gland complex) in the eyestalk acts as a negative regulator of ecdysteroidogenesis in the Y-organ (Lachaise et al., 1993; Skinner, 1985). The signal transduction pathway is poorly understood. Cyclic nucleotides mediate MIH inhibition of Y-organ ecdysteroidogenesis (Spaziani et al., 1999, 2001). There are species differences in the relative importance of cAMP and cGMP, although both cyclic nucleotides probably play a role (Lachaise et al., 1993; Sedlmeier and Fenrich, 1993; Spaziani et al., 1999). MIH induces an increase in cAMP and cGMP, with subsequent activation of protein kinases in Y-organs *in vitro* (Baghdassarian et al., 1996; Böcking and Sedlmeier, 1994; Saïdi et al., 1994; Sedlmeier and Fenrich, 1993; Von Gliscynski and Sedlmeier, 1993).

Given the wide expression of NOS in insect and mammalian tissues, we hypothesized that crustacean NOS is expressed in non-neuronal tissues and functions in regulating a variety of physiological processes. A cDNA encoding a full-length crustacean NOS (Gl-NOS) was cloned from land crab (*Gecarcinus lateralis*) Y-organ and thoracic ganglion mRNA using RT-PCR and 3′ and 5′ RACE PCR. The tissue expression of NOS mRNA was determined with RT-PCR. Immunohistochemistry was used to localize NOS protein in Y-organ, gill and ovary. The results show that NOS is more widely distributed than was previously supposed and suggest that NO is involved in regulating diverse functions, including MIH signaling in the Y-organ.

Materials and methods

Animals

Adult land crabs (*Gecarcinus lateralis* Fréminville) were collected from San Miguel Reserve near Fajardo, Puerto Rico. They were kept in covered plastic cages with aspen bedding moistened with tap water at 27°C and 30–40% humidity and were fed cat chow, carrots and lettuce twice a week. A 12 h:12 h dark:light cycle was used.

Cloning of Gl-NOS cDNA

A partial NOS cDNA was initially obtained by nested RT-PCR using degenerate primers directed to highly conserved sequences in a wide variety of *NOS* genes in the GenBank database (http://www.ncbi.nlm.nih.gov), including those from six insect species and three human forms (nNOS, eNOS, iNOS) and aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Two sets of degenerate primers were designed to anneal to DNA sequences encoding F(S/N)GWYM, VF(H/F)QEM or TFGNG(E/D)PP (Fig. 1, broken lines with solid arrowheads): NOS F1, 5′-TT(C/T) (A/T)(G/C/A)(A/G/T/C) GG(A/G/T/C) TGG TA(C/T) ATG-3′; NOS F2, 5′-GT(A/G/T/C) TT(C/T) (C/T)(A/T)(C/T) CA(G/A) GA(G/A) ATG-3′; NOS R1, 5′-GG(A/G/T/C) GG(A/G/T/C) TC(A/G/T/C) CC(G/A) TT(A/G/T/C) CC-3′; R2, 5′-G(A/G/T/C) TC(A/G/T/C) CC(G/A) TT(A/G/T/C) CC(G/A) AA(A/G/T/C) G-3′. All the primers were synthesized and purified by Integrated DNA Technologies, Inc. (Des Moines, IA, USA).

Total RNA was isolated from thoracic ganglia and Y-organ using RNeasy Protect mini kit (Qiagen, Inc., Valencia, CA, USA). About 20 mg of tissue and 600 µl of RTL reagent were used for each spin column unit. Total RNA (100 µg) was used for isolating mRNA with an Oligotex mRNA isolation kit (Qiagen). cDNA was synthesized according to the manufacturer's protocol using the Superscript II RNase H-reverse transcriptase first-strand synthesis system (Invitrogen, Inc., Carlsbad, CA, USA). Briefly, 12 µl of a mixture containing 1 µl oligo (dT)12–18 (500 µg ml^{-1}), 100 ng RNA

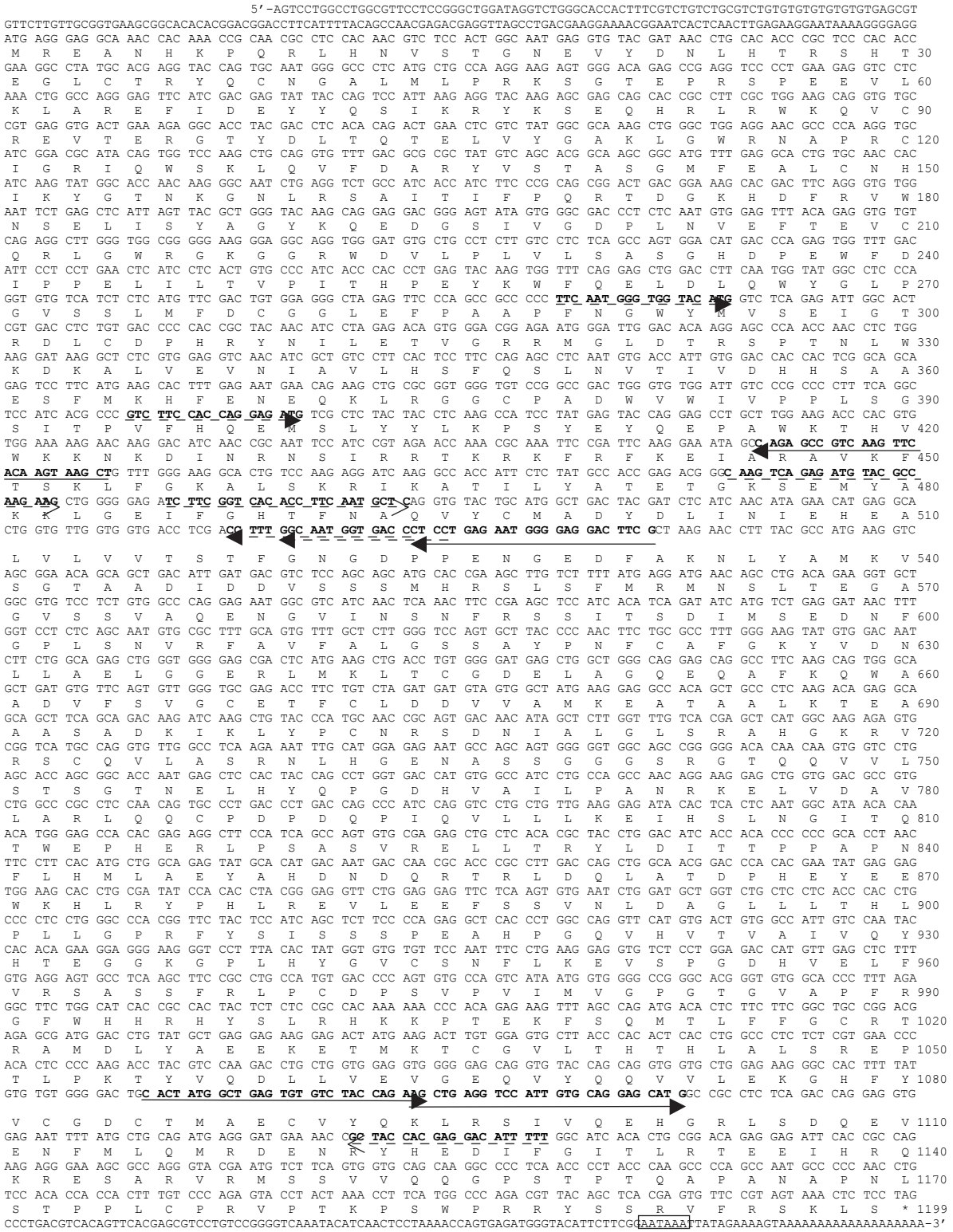

Fig. 1. The nucleotide and deduced amino acid sequences of land crab nitric oxide synthase (Gl-NOS) cDNA. The cDNA (3982 bp) contained a complete open reading frame (ORF) encoding a protein of 1199 amino acids (residue number indicated on the right). Locations and directions of degenerate primers used for nested PCR to obtain the initial cDNA are indicated by bold letters and solid arrowheads with broken lines. Locations and directions of sequence-specific forward primers and a degenerate reverse primer used for semi-nested PCR to obtain more of the 3′ ORF are indicated by open arrowheads with broken lines. Solid arrowheads with solid lines indicate locations and directions of nested sequence-specific primers used for 5′ and 3′ RACE. The poly(A) signal (AATAAA) in the 3′ UTR is boxed. GenBank accession #AY552549.

and 1 μl of 10 mmol l^{-1} dNTPs was heated to 65°C for 5 min and chilled on ice for 1 min. 4 μl of 5× First-Strand Buffer, 2 μl of 0.1 mol l^{-1} dithiothreitol (DTT) and 1 μl RNaseOUT, recombinant ribonuclease inhibitor (40 units μl^{-1}) were added. The mixture was incubated at 42°C for 2 min. The reaction was initiated by the addition of 1 μl (200 units) SuperScript II at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. PCRs were performed using an ABI 9600 thermal cycler (Perkin-Elmer, Inc., Wellesley, MA, USA). The first-round PCR contained 3 μl of cDNA, 3 μl of 10× Takara EX Taq buffer, 2 μl of 250 μmol l^{-1} dNTPs, 1 μl of forward degenerate primer (NOS F1), 1 μl of reverse degenerate primer (NOS R1), 0.2 μl of Takara EX Taq DNA polymerase (5 units μl^{-1}) and 18.8 μl of PCR-grade deionized water. Initial denaturation (95°C for 5 min) was followed by 35 amplifying cycles (95°C for 30 s, 53°C for 30 s and 72°C for 1 min) and final extension at 72°C for 7 min. For the second PCR, 0.1 μl of the first PCR reaction and the nested degenerate NOS F2 and NOS R2 primers were used. Other reaction components and PCR conditions were the same as those in the first reaction.

PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were purified from gel slices using QIAquick Gel Extraction mini kit (Qiagen), ligated into PCR2.1 vector with the TOPO TA Cloning kit (Invitrogen) and transformed into One Shot TOP 10 *E. coli* strain (Invitrogen). Plasmids were purified using Qiagen spin mini prep kit and sequenced using T7 and M13 reverse vector primers (Davis Sequencing, Davis, CA, USA).

A semi-nested RT-PCR strategy was used to obtain more of the ORF 3′ to the initial nested RT-PCR product. The reactions used two sequence-specific forward primers (cNOS F5, 5′-CAAGTCAGAGATGTACGCCAAGAAG-3′, and cNOS F6, 5′-TCTTCGGTCACACCTTCAATGCTC-3′) and a degenerate reverse primer (NOS R3, 5′-RAADATRTCYT-CRTGRTANC-3′) to a highly conserved sequence in the reductase domain (Fig. 1, broken lines with open arrowheads). First-round PCR used cNOS F5 and NOS R3 primers and the original cDNA synthesized from thoracic ganglia and Y-organ mRNA (see above). Second-round PCR used cNOS F6 and NOS R3 primers and 0.1 μl of the first-round PCR. The PCR conditions were the same as described above, except that the extension time for the amplification cycles was 2 min instead of 1 min. PCR products were separated on 1% agarose gels, purified, cloned and sequenced as described above.

RACE (rapid amplification of cDNA ends) of mRNA was used to obtain full-length sequences. Poly(A^+) RNA was isolated from total RNA using Oligotex mRNA kit (Qiagen). For the 3′ sequence, the RACE System (Invitrogen) was used. Briefly, first-strand cDNA synthesis reactions contained 200 ng poly(A^+) RNA and adaptor primer (5′-GGC-CACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′). First-round PCR on the cDNA (20 ng) included a universal amplification primer (5′-CUACUACUACUAGGCCACGC-GTCGACTAGTAC-3′) and gene-specific forward primer, cNOS F1 (5′-CACTATGGCTGAGTGTGTCTACCAGAAG-3′), under the following conditions: denaturation at 96°C for 5 min, 35 amplification cycles (96°C for 30 s, 60°C for 30 s and 72°C for 2 min) and final extension at 72°C for 10 min. Nested PCR (30 μl total volume) was conducted with a gene-specific primer, cNOS F2 (5′-AGCTGAGGTCCATTGT-GCAGGAGCATG-3′), and an abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) under the same conditions as the first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

The SMART™ RACE cDNA amplification kit (BD Biosciences, Inc., San Jose, CA, USA) was used to obtain the 5′ sequence. The first-strand cDNA synthesis reaction contained 3 μl poly(A+) RNA (100 ng), 1 μl 5′ CDS primer [10 mmol l^{-1}, 5′-(T)25N-1N-3′] and 1 μl SMART II A oligo (10 mmol l^{-1}, 5′-AAGCAGTGGTATCAACGCAGAGTAC-GCGGG-3′) and was incubated at 68°C for 2 min. After cooling the reaction on ice for 2 min, 2 μl of 5× First-Strand buffer [250 mmol l^{-1} Tris-HCl (pH 8.3), 375 mmol l^{-1} KCl and 30 mmol l^{-1} $MgCl_2$], 1 μl DTT (20 mmol l^{-1}), 1 μl dNTPs (10 mmol l^{-1}) and 1 μl PowerScript Reverse Transcriptase were added. The reaction was covered with 20 μl paraffin oil and incubated at 42°C for 1.5 h in an ABI 9600 DNA thermal cycler (Perkin-Elmer). The reaction mixture was diluted 10-fold with autoclaved distilled water and was used for first-round PCR with 10× universal primer A mix (0.4 mmol l^{-1} 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTAT-CAACGCAGAGT-3′ and 2 mmol l^{-1} 5′-CTAATACGACT-CACTATAGGGC-3′) and gene-specific reverse primer, cNOS R1 (5′-CGAAGTCCTCCCCATTCTCAGGAG-3′), under the following conditions: denaturation at 96°C for 5 min, 35 amplification cycles (96°C for 30 s, 66°C for 15 s and 72°C for 3 min) and final extension at 72°C for 10 min. Second-round PCR was conducted using nested gene-specific primer cNOS R2 (5′-AGCTTACTTGTGAACTTGACGGCTCTG-3′) and nested universal primer A (10 mmol l^{-1}, 5′-AAGCAGTG-GTATCAACGCAGAGT-3′). The PCR conditions were the same as those used for first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Purified products were sequenced to confirm identities.

Gl-NOS expression by RT-PCR

Integument, thoracic ganglia, testis, ovary, heart, digestive gland, gill, claw muscle, eyestalk neural ganglia and Y-organ were dissected from 3–5 crabs and immediately placed in RNAlater RNA stabilization reagent (Qiagen). Tissues were stored at –20°C until RNA extractions could be performed. Total RNA was isolated from pooled tissues using either the RNeasy mini or midi kit according to the manufacturer's instructions (Qiagen). RNA concentration was determined by UV absorbance at 260 nm and stored at –80°C. About 1 μg of total RNA was used for the reverse transcription reaction. RNA was first treated with DNase I to degrade any contaminating genomic DNA. First-strand cDNA was synthesized in a 20 μl reaction volume containing 50 mmol l^{-1} Tris-HCl, 75 mmol l^{-1}

KCl, 3 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} DTT, 0.5 mmol l^{-1} of each dNTP, 40 units of RNaseOUT ribonuclease inhibitor, 1 ng oligo dT primer and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction was incubated for 50 min at 37°C, heat-inactivated and stored at –20°C.

The quality of the cDNA was first verified by performing PCR with land crab elongation factor 2 (EF2; GenBank accession #AY552550) primers (cEF F1, 5′-TTCTATGCCTTTGGCCGTGTCTTCTC-3′; cEF R1, 5′-TGATGGTGCCCGTCTTAACCAGATAC-3′). The PCR conditions were an initial denaturation at 95°C for 2 min, 35 amplification cycles (denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s) and 2 min at 72°C as a final extension.

NOS PCR was then performed in a 20-μl reaction mixture as described above using 2 μl of the first-strand cDNA and an *NOS* gene-specific primer pair (cNOS EXF, 5′-CAACTTGAGAAGGAATAAAAGGGGAGGATG-3′; cNOS R31, 5′-CTGCTGAAGCTGCTGCCTCTGTCTTGAG-3′), each at a final concentration of 0.2 μmol l^{-1}. The PCR conditions were an initial denaturation at 95°C for 2 min, 35 amplification cycles (denaturation at 96°C for 20 s, annealing at 62°C for 20 s and extension at 72°C for 90 s) and 4 min at 72°C as a final extension. This primary PCR reaction was then used as template with a nested NOS primer pair (cNOS F1, 5′-GTACAAGCAGGAGGACGGGAG-3′; cNOS R5, 5′-AGCTTACTTGTGAACTTGACGGCTCTG-3′), each at a final concentration of 0.2 μmol l^{-1} as described above. The primary reaction was diluted 1:10 000 in water, and 2 μl was used in the reaction. The PCR conditions were an initial denaturation at 95°C for 2 min, 35 amplification cycles (denaturation at 96°C for 20 s, annealing at 62°C for 20 s and extension at 72°C for 50 s) and 4 min at 72°C as a final extension. All PCR reactions were analyzed by separating some or all of the 20 μl reaction volume on 1–2% agarose gels with a 100 bp PCR Molecular Ruler DNA size ladder (Bio-Rad, Inc., Hercules, CA, USA).

Immunohistochemistry

Tissues used for immunohistochemistry were dissected and fixed for 24–48 h in Bouin's fixative or Histochoice MB fixative (Amresco, Inc., Solon, OH, USA) containing 0.15 mol l^{-1} NaCl. The tissue was then dehydrated through a graded ethanol series, cleared and embedded in paraffin. Sections (~9 μm) were placed on clean glass slides and allowed to dry at 40°C for 24 h. Sections were then deparaffinized in xylene and rehydrated through an ethanol series into a phosphate-buffered saline (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 4.3 mmol l^{-1} Na_2HPO_4, 1.4 mmol l^{-1} KH_2PO_4; pH 7) with 15 mmol l^{-1} glycine (PBSG). Sections were incubated at room temperature in 1% bovine serum albumin (BSA) and normal goat serum in PBSG for 45 min and then with primary antibody (rabbit anti-NOS antibody; Affinity BioReagents, Inc., Golden, CO, USA; 1:1000–2000 dilution in PBSG) at 4°C overnight. The NOS antibody was a universal antibody (generated against a portion of murine nNOS/iNOS) that crossreacts with crustacean NOS (Christie et al., 2003; Scholz et al., 1998, 2002). After four rinses (5 min each) in PBSG, sections were incubated with either a goat anti-rabbit or mouse anti-rabbit biotin-conjugated antibody (Pierce, Inc., Rockford, IL, USA; diluted 1:1000–1500) at room temperature for 2 h, rinsed four times in PBSG and incubated with an avidin/alkaline phosphatase reagent (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were rinsed four times in PBSG and incubated with BCIP/NBT (Gibco/Invitrogen) or Vector Red (Vector Laboratories, Inc.) substrate until sufficient color development was attained. Control incubations were also done on adjacent tissue sections and included either omitting the primary antibody or substituting a non-immune rabbit serum for the primary antibody.

Results

Cloning and tissue expression of Gl-NOS cDNA

Nested PCR using NOS F2 and R2 primers amplified an initial product of ~400 bp. The deduced amino acid sequence had 72% identity with the CaM-binding and flavodoxin-like regions of *Drosophila* NOS (accession number AAC46882). A cDNA (1913 bp) was amplified by semi-nested PCR to obtain more sequence 3′ to the initial 400-bp product. The deduced amino acid sequence of the 1913-bp product was 50% identical to that of NOS from the insect *Rhodnius prolixus* (accession number Q26240). A 1.5-kb product containing the 5′ UTR and the remainder of the 5′ ORF was obtained from 5′ RACE PCR using a sequence-specific reverse primer (cNOS R2; Fig. 1). A 500-bp product containing the 3′ UTR and the remainder of the 3′ ORF was obtained from 3′ RACE PCR using a sequence-specific forward primer (cNOS F2; Fig. 1). The combined PCR products contained a full-length 3982-bp sequence of land crab NOS cDNA (Gl-NOS; GenBank accession #AY552549).

The Gl-NOS cDNA encoded a protein containing 1199 amino acids with an estimated mass of 135 624 Da (Fig. 1). The Gl-NOS amino sequence was aligned with NOS sequences from five insects, a mollusk and three human types (Fig. 2). The N-terminal region varied among different *NOS* genes, but the oxygenase domain in *Gl-NOS* was 70% identical to the *Drosophila NOS*, 68% identical to *Aplysia NOS* and 66% identical to human *nNOS*. In the oxygenase domain, the heme-binding motif was well conserved, including the cysteine residue that acts as an axial ligand. The motifs for binding tetrahydrobiopterin (H4) cofactor and CaM were also well-conserved. The reductase domain contained all conserved binding motifs for FMN, FAD and NADPH. Interestingly, the Gl-NOS had single amino acid substitutions in all three motifs for binding FAD that differed from other NOS cDNAs (Fig. 2). The domain organization of *Drosophila* NOS and Gl-NOS is compared in Fig. 3 to show the high amount of similarity between the two sequences.

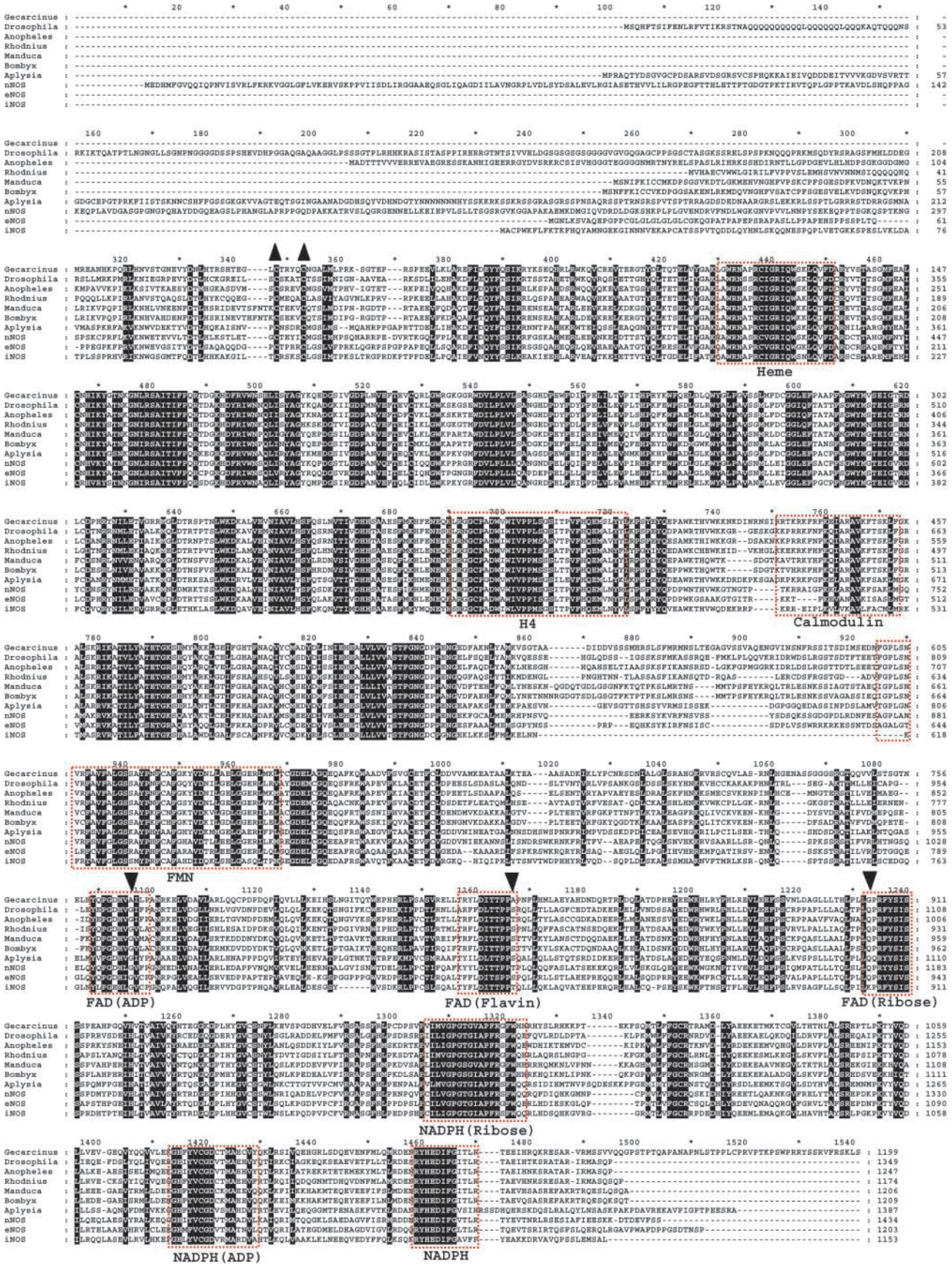
Gecarcinus
Drosophila
Anopheles
Rhodnius
Manduca
Bombyx
Aplysia
nNOS
eNOS
iNOS
Heme
H4
Calmodulin
FMN
FAD(ADP)
FAD(Flavin)
FAD(Ribose)
NADPH(Ribose)
NADPH(ADP)
NADPH

Fig. 2.

Fig. 2. Comparison of deduced amino acid sequences of NO synthase from land crab, insects, mollusk and human. Land crab (*Gecarcinus lateralis*) NOS was aligned with NOS sequences from insects (*Manduca sexta*, *Bombyx mori*, *Rodnius prolixus*, *Anopheles stephensi* and *Drosophila melanogaster*), mollusk (*Aplysia californica*) and human (iNOS, nNOS, eNOS) using the ClustalW program (see Materials and methods). Identities in all 10 sequences are highlighted in black. Boxes with broken borders identify highly conserved binding sequences for heme, tetrahydrobiopterin (H4), calmodulin, FMN, FAD and NADPH. Inverted triangles indicate amino acid sequence deviation in the FAD binding motif of Gl-NOS. Regular triangles indicate the two conserved cysteine residues in the zinc tetrathiolate cluster. Accession numbers: *Rhodnius prolixus*, Q26240; *Anopheles stephensi*, O61608; *Bombyx mori*, BAB85836; *Drosophila melanogaster*, Q27571; *Manduca sexta*, T30555; *Aplysia californica*, AF288780; and human iNOS (AAB49041), eNOS (NP000594) and nNOS (NP000611).

The phylogenetic relationships of various NOS sequences were determined using sequence alignments of the oxygenase domains (Fig. 4; residues #54–455 in Gl-NOS). Insect NOS sequences clustered according to major taxonomic groups: Lepidoptera (*M. sexta* and *B. mori*), Diptera (*A. stephensi* and *D. melanogaster*) and Hemiptera (*R. prolixus*). Molluskan NOS (*A. californica*) and vertebrate NOS formed distinct groups. Within the vertebrates, the inducible NOS (iNOS) and noninducible NOS (nNOS, eNOS) were divided. Since few *NOS* genes have been obtained from lower invertebrates (e.g. nematode), Gl-NOS could not be grouped with any other NOS, although overall sequence comparison showed that Gl-NOS was most closely related to *Drosophila* NOS.

Gl-NOS was expressed in both neuronal and non-neuronal tissues. Initial RT-PCR using cNOS EXF and R31 primers generated a 2110-bp product amplified from RNA isolated from testis, gill, ovary, eyestalk neural ganglia and Y-organ (Fig. 5A, lanes c, f–i). RT-PCR with an elongation factor 2 (EF2) primer pair served as a positive control (Fig. 5C). Nested PCR on the first-round PCR product using cNOS F1 and R5 primers generated a product of the expected size (795 bp), which confirmed the identity of the initial product as the NOS sequence (Fig. 5B). Gl-NOS mRNA varied in Y-organ, thoracic ganglion and gill; in some preparations, no PCR product was detected in these tissues. Although no PCR product was obtained from the thoracic ganglion mRNA preparation in Fig. 5 (lane b), other thoracic ganglion mRNA preparations yielded an NOS PCR product (data not shown). By contrast, the NOS mRNA was present at consistently high levels in all RNA preparations from eyestalk ganglia, ovary and testis.

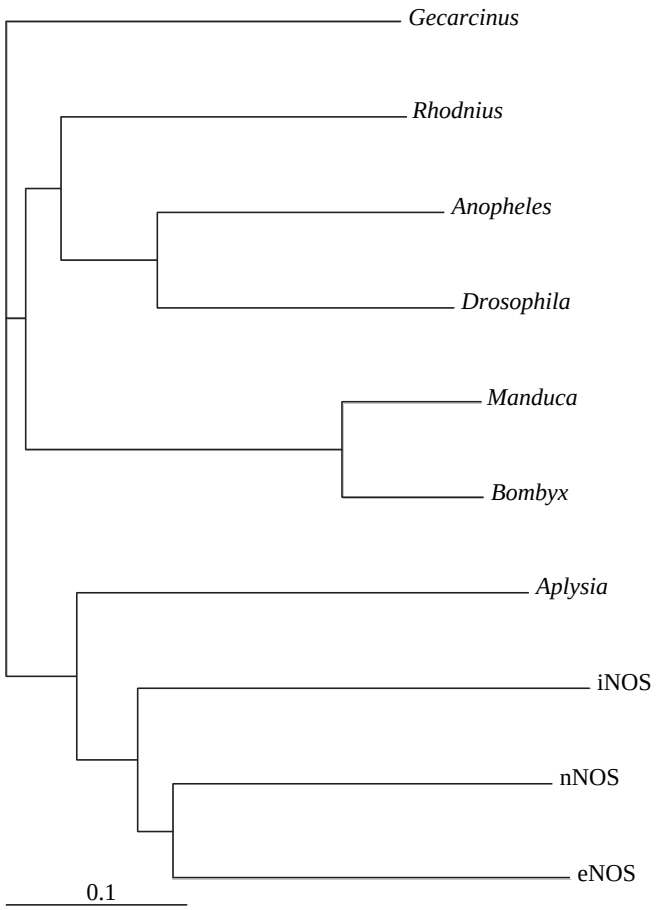

Fig. 4. Phylogenetic relationships of NO synthases from arthropods, mollusk and mammals. The deduced amino acid sequences of the oxygenase domain were analyzed using ClustalW and Treeview programs. Arthropod NOS sequences form a group divergent from molluscan and mammalian NOS sequences. Within the arthropods, the land crab NOS was diverged from insects. Within Insecta, NOS sequences grouped according to major taxonomic lineages: Lepidoptera (*Manduca* and *Bombyx*), Diptera (*Drosophila* and *Anopheles*) and Hemiptera (*Rhodnius*). Accession numbers were the same as those given in the legend for Fig. 2.

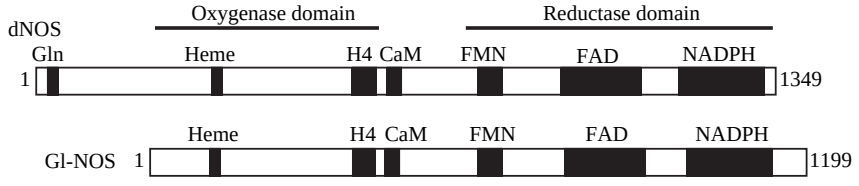

Fig. 3. Domain organization of arthropod NOS. The ORFs of *Drosophila* (dNOS) and land crab NOS (Gl-NOS) are compared. The oxygenase domain contains heme-binding and tetrahydrobiopterin (H4) domains; the reductase domain contains binding domains for FMN, FAD and NADPH. A calmodulin (CaM) binding domain is located between the oxygenase and reductase domains and is involved in dimerization and regulation of catalytic activity (Regulski and Tully, 1995; Stasiv et al., 2001). dNOS has a Gln-rich sequence near the amino terminus.

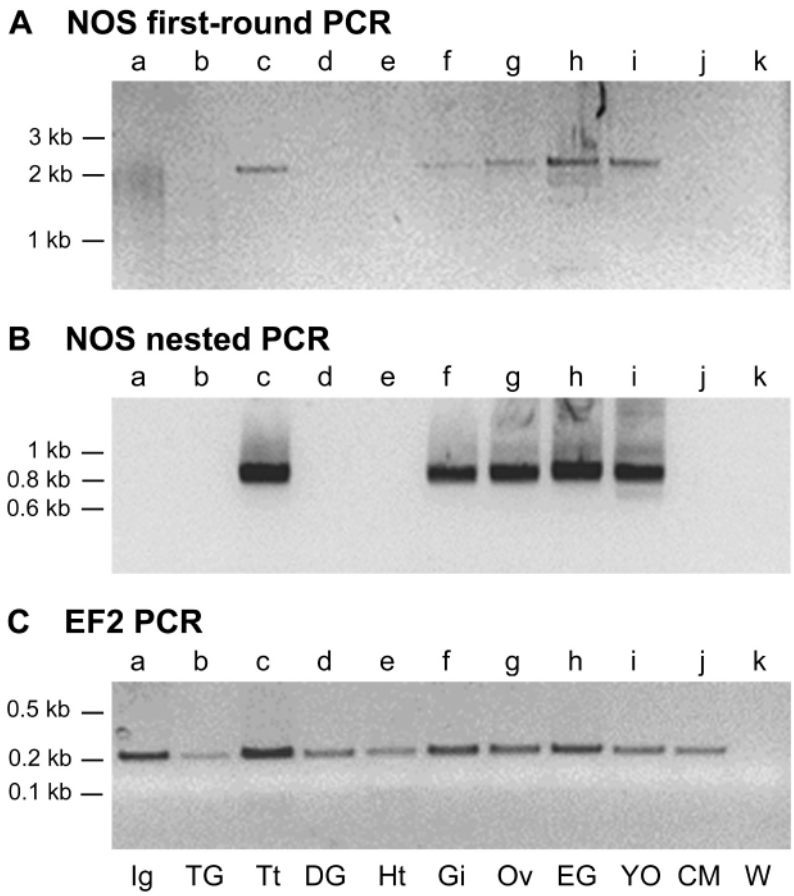

Fig. 5. Expression of NO synthase (Gl-NOS) in land crab tissues. Total RNA was DNase-treated, reverse-transcribed and PCR-amplified using sequence-specific primers (see Materials and methods). Shown are inverse images of ethidium bromide-stained agarose gels of the PCR products. (A) First-round PCR generated a product of the expected size (~2.1 kb). Gl-NOS was expressed in testis (Tt), gill (Gi), ovary (Ov), eyestalk neural ganglia (EG) and Y-organ (YO). Gl-NOS mRNA was not detected in integument (Ig), thoracic ganglion (TG), digestive gland (DG), heart (Ht) or claw muscle (CM). (B) Nested PCR of the initial PCR generated a product of the expected size (~800 bp), which confirmed the identity of the initial product as Gl-NOS. In other experiments, a Gl-NOS product was obtained from thoracic ganglion (data not shown). (C) Elongation factor 2 (EF2) served as internal positive control, as it was constitutively expressed in all tissues. Reactions without template [water (W) lane k] served as a negative control. Positions of DNA size markers are indicated on the left.

Immunohistochemistry

Immunohistochemistry was used to confirm that the NOS protein is present in Y-organs and other non-neuronal tissues. A pair of Y-organs is located adjacent to the branchial chamber on one side and a hemolymph sinus and connective tissue on the other. A thin cuticle separates the Y-organ from the branchial chamber. A universal anti-NOS antibody reacted with the nuclei and cytoplasm of all Y-organ cells (Figs 6A, 7A). Some nuclei in the connective tissue (Fig. 6A,C,D) were stained, as well as the tendinous cells, which anchor connective tissue to the cuticle lining the branchial chamber (Fig. 6D).

In gill, the NOS protein was localized in the epithelium (Fig. 7B). Staining was more intense in the epithelium lining the central axis between the gill lamellae (Fig. 7B, arrows) and in the pillar cells (Fig. 7B, arrowheads). In the ovary, the NOS protein was confined to the perinuclear cytoplasm of oocytes (Fig. 7D). Control sections without primary antibody showed a low amount of staining in oocytes, indicating some non-specific binding of the biotinylated secondary antibody and/or the avidin/alkaline phosphatase reagent (Fig. 7C).

Discussion

NOS produces NO by catalyzing the conversion of L-arginine to L-citrulline and oxidation of NADPH. Three types of NOS are found in vertebrates (Bogdan, 2001; Kone, 2001). Both nNOS and eNOS are Ca^{2+}/CaM-dependent, whereas iNOS is Ca^{2+}-independent. All are homodimers consisting of a 130–160-kDa subunit. Invertebrates appear to have only a Ca^{2+}/CaM-dependent form (Davies, 2000; Korneev et al., 1998; Luckhart and Rosenberg, 1999; Nighorn et al., 1998; Regulski and Tully, 1995; Ribeiro and Nussenzveig, 1993; Stasiv et al., 2001). We have cloned a cDNA encoding a full-length sequence of land crab NOS (Gl-NOS) from Y-organ and thoracic ganglion mRNA (Fig. 1). The ORF codes for a 1199 amino acid protein with an estimated mass of ~136 kDa, which is similar to the mass of a 138-kDa NOS-immunoreactive protein in crayfish eyestalk neural ganglia extract (Lee et al., 2000). The domain organization is highly conserved, with a CaM-binding domain located between the oxygenase and reductase domains (Figs 2, 3). Interestingly, Gl-NOS has single amino acid changes in all three FAD binding motifs (Fig. 2). In the FAD(ADP) site, the Gl-NOS has an alanine (A), whereas all the other sequences have a glycine (G) in that location. This is a conservative substitution, as both alanine and glycine are nonpolar residues. The differences in the other two sites are greater, as a nonpolar residue in Gl-NOS replaces a polar residue in the other NOS sequences. An alanine replaces a serine (S) or threonine (T) in the FAD(flavin) site, and glycine (G) replaces a lysine (K) or glutamine (Q) in the FAD(ribose) site. Since the FAD binding motif participates in

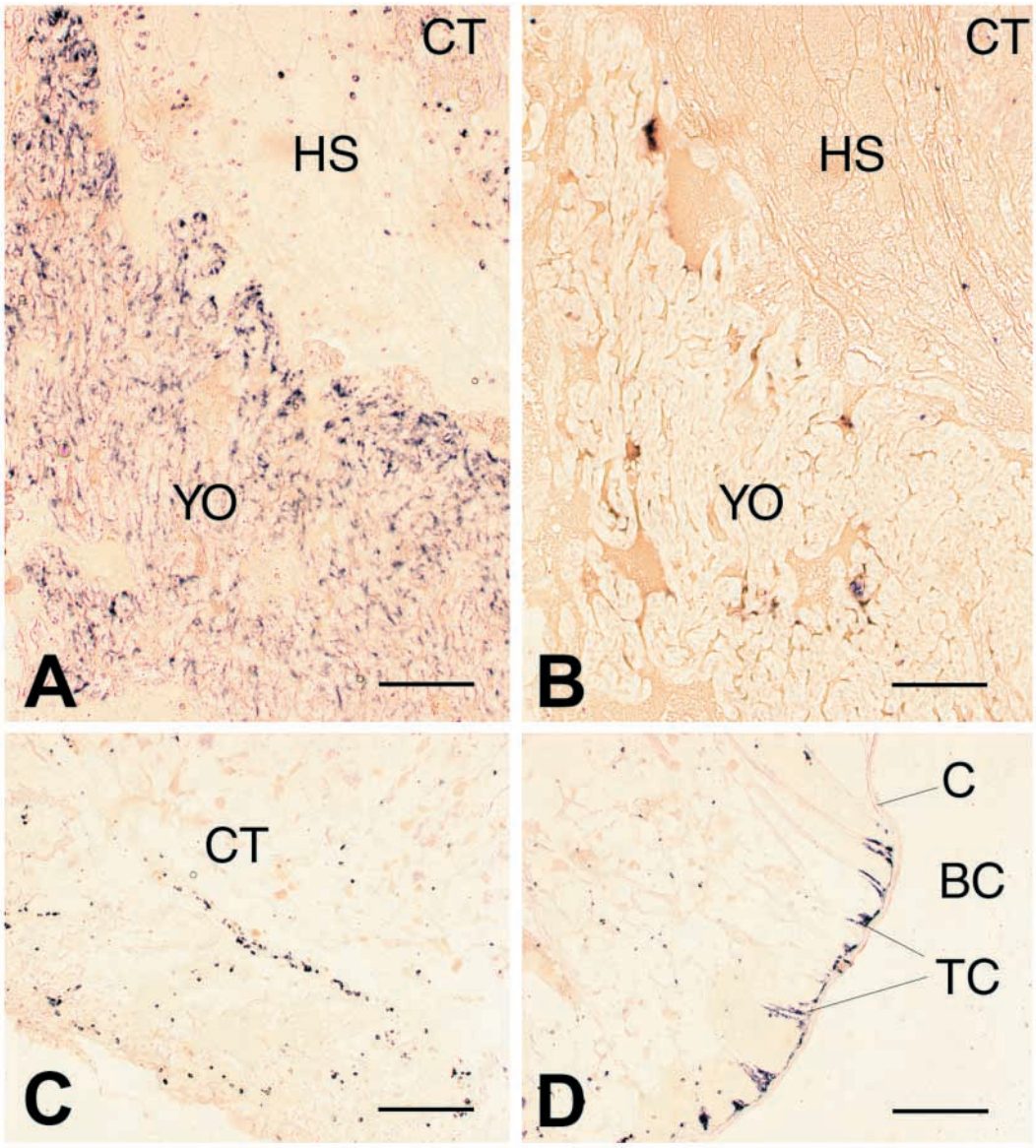

Fig. 6. Immunocytochemical localization of NO synthase in land crab Y-organs. Sections of Y-organ were incubated with either a universal anti-NOS rabbit antibody (A, C and D; 1:2000 dilution) or a non-immune rabbit antibody (B; 1:2000 dilution); detection used BCIP/NBT (see Materials and methods). (A) NOS localization in the cytoplasm and nuclei of Y-organ (YO) cells and nuclei in some cells in the adjacent hemolymph space (HS) and connective tissue (CT). (B) Control serial section adjacent to A showing no specific antibody binding in Y-organ tissue. (C) NOS localization in the nuclei of some connective tissue cells. (D) NOS localization in the tendinous cells (TC), which anchor connective tissue to the cuticle (C). BC, branchial chamber. Scale bars, 200 μm.

electron transfer within the reductase domain, these changes may affect NO production by altering the binding affinity for FAD.

NOS genes are regulated at the transcriptional, translational and post-translational levels (Hall et al., 1994; Kone, 2001; Wang et al., 1999). Numerous isoforms of human nNOS are produced by alternative promoters and alternative splicing and differ in tissue expression patterns (Kone, 2001; Wang et al., 1999). Multiple isoforms of *Drosophila* NOS (dNOS) result from alternative mRNA splicing; the truncated isoforms lack NOS activity and those that have sequences required for dimerization in the vicinity of the CaM-binding domain act as dominant negative regulators (Regulski and Tully, 1995; Stasiv et al., 2001). The dNOS isoforms are differentially expressed during *Drosophila* development (Stasiv et al., 2001). In the mosquito *Anopheles stephensi*, 18–22 NOS alternative transcripts are expressed (Luckhart and Li, 2001). The number of alternative transcripts expressed in land crab has not been determined. Our preliminary results indicate that a truncated isoform was expressed in the Y-organ. In this cDNA, alternative splicing introduced a stop codon in the reductase domain, resulting in a polypeptide of 720 amino acids in

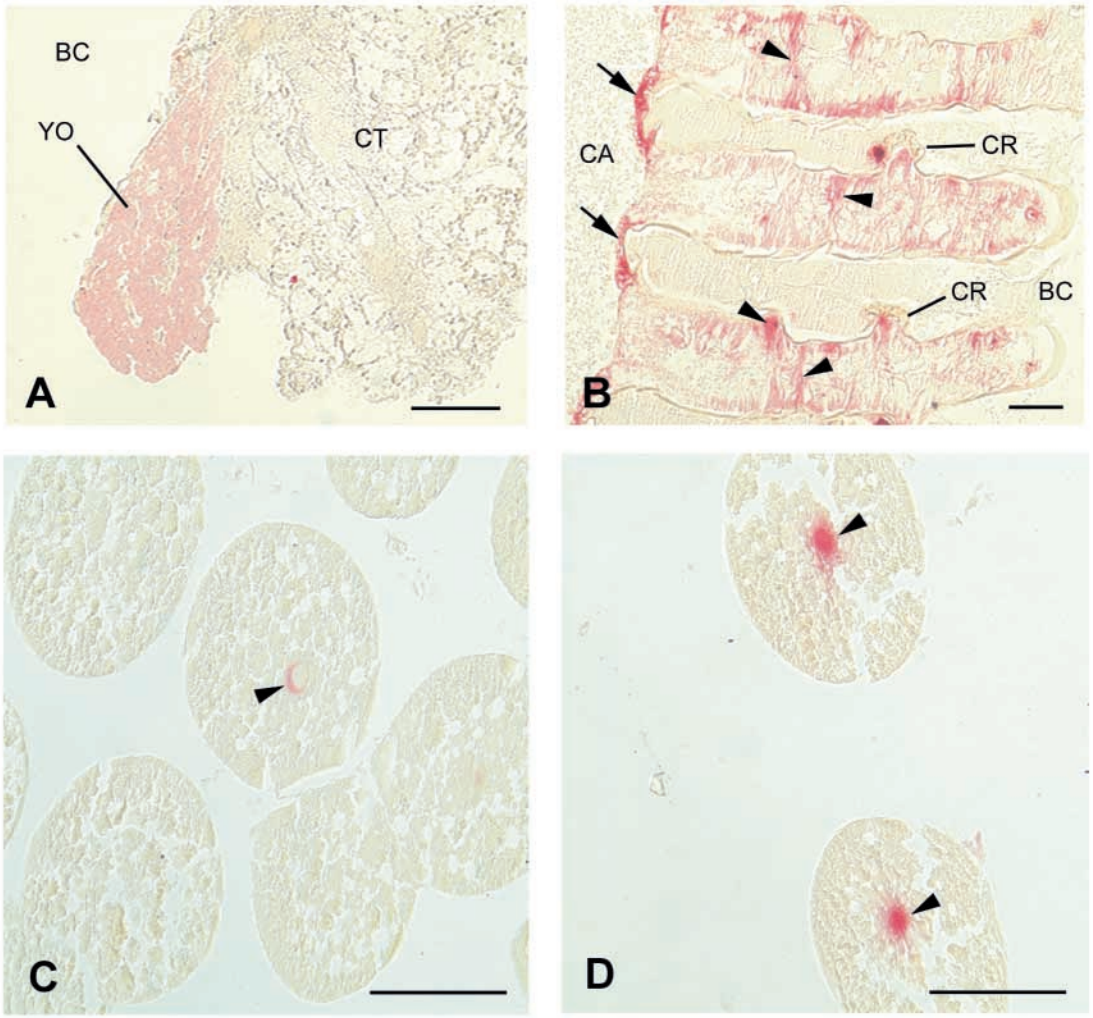

Fig. 7. Immunocytochemical localization of NO synthase in Y-organ, gill and ovary. Sections were incubated with a universal anti-NOS rabbit antibody (1:1000 dilution; A, B and D) or no primary antibody (C); detection used Vector Red (see Materials and methods). (A) Y-organ (YO). NOS was localized in cytoplasm and nuclei of YO cells. Connective tissue (CT) showed little or no staining. BC, branchial chamber. (B) Gill. The field includes a portion of the central axis (CA) and transverse sections through three lamellae. NOS was localized in the epithelium and pillar cells (arrowheads). The epithelium lining the central axis stained more intensely (arrows). Control sections of Y-organ and gill incubated with either non-immune primary antibody or no primary antibody showed no specific staining (data not shown). Cuticular ridges (CR) keep lamellar surfaces from touching, creating a space for air circulation between gill lamellae. (C) Ovary control (no primary antibody), showing weak non-specific staining in the perinuclear region of oocytes (arrowhead). (D) Ovary. NOS was confined to the perinuclear cytoplasm of oocytes (arrowheads). Scale bars: 400 μm in A, 50 μm in B, and 200 μm in C and D.

length. This isoform may act as a dominant negative regulator, as it retains the dimerization sequence but lacks a complete reductase domain. It was not characterized further, because it was expressed at low levels in the Y-organ and thoracic ganglion.

In insects, the NO/cGMP signaling pathway is involved in such diverse functions as phototransduction, olfaction, neuronal development, ecdysis, food search behavior and epithelial fluid transport (Davies, 2000; Morton and Hudson, 2002). Until recently, NOS had only been reported in the nervous system of crustaceans, in which it is involved in regulating neuronal activity (Aonuma et al., 2000; Aonuma and Newland, 2002; Johansson and Carlberg, 1994; Johansson and Mellon, 1998; Lee et al., 2000; Scholz et al., 1998, 2001, 2002; Schuppe et al., 2001a,b, 2002; Talavera et al., 1995; Zou et al., 2002). In lobster, NO produced by the cardiac muscle reduces bursting activity of the cardiac gangion, which decreases heartbeat amplitude and frequency (Mahadevan et al., 2004). In addition to nervous tissue, we have shown that the land crab Y-organ, gill, testis and ovary express NOS by RT-PCR (Fig. 5) and immunohistochemistry (Figs 6, 7). However, we did not detect NOS mRNA in land crab heart, even after a second round of PCR with nested primers (Fig. 5B). Although this is the first report of NOS in these tissues from a crustacean, non-neuronal NOS has been reported in insect Malpighian tubules, imaginal discs and salivary glands (Davies et al., 1997;

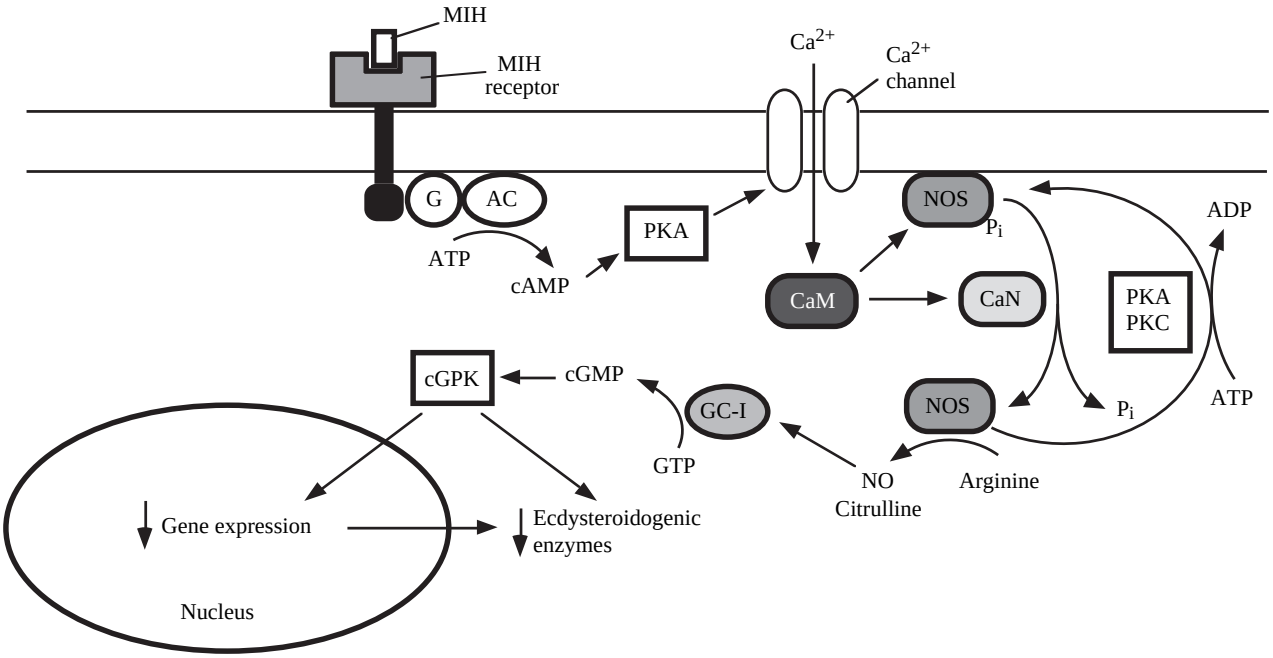

Fig. 8. Hypothetical signaling pathway inhibiting ecdysteroidogenesis in the crustacean Y-organ. Molt inhibiting hormone (MIH) binds to a G protein-coupled receptor (G), activating adenylyl cyclase (AC); intracellular Ca^{2+} rises when protein kinase A (PKA) activates a membrane Ca^{2+} channel; nitric oxide synthase (NOS) is activated and released from the membrane when Ca^{2+}/calmodulin (CaM) binds, and NOS is dephosphorylated by the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (CaN); NO activates an NO-sensitive (class I) GC (GC-I). Activation of a cGMP-dependent protein kinase (cGPK) inhibits expression and/or activities of ecdysteroidogenic proteins, resulting in reduced ecdysteroid synthesis. NOS is inactivated by phosphorylation by PKA, protein kinase C (PKC) or other protein kinases.

Kuzin et al., 1996, 2000; Ribeiro and Nussenzveig, 1993; Stasiv et al., 2001).

The presence of NOS in crustacean non-neuronal tissues suggests that NO signaling is involved in physiological processes in addition to neuromodulation. NOS was not detected in skeletal muscle (Fig. 5), although NO and cGMP increase inward Ca^{2+} current and both early and delayed outward K^+ currents in skeletal muscle of a marine isopod (Erxleben and Hermann, 2001; Hermann and Erxleben, 2001). The functions of NOS in Y-organ, ovary, testis and gill are not known. In gonadal tissue, NOS may regulate gametogenesis and/or steroidogenesis. In blowfly, for example, steroid synthesis is inhibited by NO and cGMP. However, crustacean ovary accumulates ecdysteroid, but there is little evidence that it is a significant site for ecdysteroid synthesis (Gunamalai et al., 2003; Spaziani et al., 1997; Subramoniam, 2000; Suzuki et al., 1996; Warrier et al., 2001). The localization of NOS in connective tissue and epithelia in Y-organ and gill suggests that it functions in an immune response to pathogens. In insects, hemocytes express an NOS that is activated upon bacterial infection (Weiske and Wiesner, 1999). *Plasmodium* infection in mosquitoes leads to a rapid induction of NOS activity (Luckhart et al., 1998). The pillar cells, which extend across each lamella and anchor in the cuticle, restrict distension caused by hemolymph pressure (Copeland, 1968; Taylor and Taylor, 1992). The localization of NOS in tendinous and pillar cells suggests that NOS is involved in the regulation of blood pressure, as it is in vertebrates.

The presence of NOS in the Y-organ suggests that NO regulates ecdysteroidogenesis *via* the activation of a soluble NO-sensitive GC-I. A hypothetical pathway is presented in Fig. 8, which is consistent with the available data. Increased cAMP, cGMP and Ca^{2+} levels inhibit ecdysteroid synthesis in crustacean Y-organ (Spaziani et al., 1999, 2001). We propose that MIH binds to a G protein-coupled receptor, leading to activation of NOS by the combined effects of dephosphorylation by calcineurin and binding of CaM. The regulation of NOS by phosphorylation/dephosphorylation is complex. Several protein kinases phosphorylate NOS in different regions (Bredt et al., 1992), which may have different effects on enzyme activity (Kone, 2001; Nakane et al., 1991). For example, CaM kinase II phosphorylates nNOS at Ser847, which inhibits enzyme activity by reducing its binding affinity for CaM (Hayashi et al., 1999; Komeima et al., 2000). The NO activates a GC-I, resulting in an increase in cGMP. This is similar to the signaling mechanism that stimulates fluid secretion in *Drosophila* Malpighian tubules by the decapeptide cardioacceleratory peptide 2b (Davies, 2000; Davies et al., 1995, 1997; Dow et al., 1994; Kean et al., 2002; MacPherson et al., 2001; Rosay et al., 1997) and inhibits steroidogenesis in blowfly ovary (Maniere et al., 2003). We have recently cloned a cDNA encoding a GC-I that is expressed in land crab Y-organ (H.-W.K. and D.L.M., data not shown). Studies are now

in progress to determine the role of NOS and GC-I in the MIH signaling pathway.

This research was supported by a grant from the National Science Foundation (IBN-9904528). L.A.B. was supported in part by a Research Experience for Undergraduates supplement from NSF. We thank Hector C. Horta (Puerto Rico Department of Natural Resources) and Hector J. Horta for collecting land crabs; Marian Allen for technical assistance; Laura Baker, Emma James, Stephanie Spenny and Kristin Van Ort for animal care; and Dr Scott Medler for critical reading of the manuscript.

References

Ahern, G. P., Klyachko, V. A. and Jackson, M. B. (2002). cGMP and *S*-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends Neurosci.* **25**, 510-517.

Aonuma, H. and Newland, P. L. (2001). Opposing actions of nitric oxide on synaptic inputs of identified interneurones in the central nervous system of the crayfish. *J. Exp. Biol.* **204**, 1319-1332.

Aonuma, H. and Newland, P. L. (2002). Synaptic inputs onto spiking local interneurons in crayfish are depressed by nitric oxide. *J. Neurobiol.* **52**, 144-155.

Aonuma, H., Nagayama, T. and Takahata, M. (2000). Modulatory effects of nitric oxide on synaptic depression in the crayfish neuromuscular system. *J. Exp. Biol.* **203**, 3595-3602.

Baghdassarian, D., De Bessé, N., Saïdi, B., Sommé, G. and Lachaise, F. (1996). Neuropeptide-induced inhibition of steroidogenesis in crab molting glands: involvement of cGMP-dependent protein kinase. *Gen. Comp. Endocrinol.* **104**, 41-51.

Baranano, D. E. and Snyder, S. H. (2001). Neural roles for heme oxygenase: contrasts to nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **98**, 10996-11002.

Bicker, G. (2001). Sources and targets of nitric oxide signalling in insect nervous systems. *Cell Tissue Res.* **303**, 137-146.

Böcking, D. and Sedlmeier, D. (1994). Protein phosphorylation in the molting gand of the crayfish, *Orconectes limosus* – role of cyclic nucleotides, calcium, and molt inhibiting hormone (MIH). *Invert. Reprod. Dev.* **26**, 237-245.

Bogdan, C. (2001). Nitric oxide and the regulation of gene expression. *Trends Cell Biol.* **11**, 66-75.

Bredt, D. S. and Snyder, S. H. (1994). Nitric oxide: a physiological messenger molecule. *Annu. Rev. Biochem.* **63**, 175-195.

Bredt, D. S., Ferris, C. D. and Snyder, S. H. (1992). Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein-kinase; identification of flavin and calmodulin binding sites. *J. Biol. Chem.* **267**, 10976-10981.

Broderick, K. E., MacPherson, M. R., Regulski, M., Tully, T., Dow, J. A. T. and Davies, S. A. (2003). Interactions between epithelial nitric oxide signaling and phosphodiesterase activity in *Drosophila*. *Am. J. Physiol.* **285**, C1207-C1218.

Chen, P. F. and Wu, K. K. (2003). Structural elements contribute to the calcium/calmodulin dependence on enzyme activation in human endothelial nitric-oxide synthase. *J. Biol. Chem.* **278**, 52392-52400.

Christie, A. E., Edwards, J. M., Cherny, E., Clason, T. A. and Graubard, K. (2003). Immunocytochemical evidence for nitric oxide- and carbon monoxide-producing neurons in the stomatogastric nervous system of the crayfish *Cherax quadricarinatus*. *J. Comp. Neurol.* **467**, 293-306.

Colasanti, M. and Venturini, G. (2000). Nitric oxide in invertebrates. *Molec. Neurobiol.* **17**, 157-174.

Copeland, D. E. (1968). Fine structure of salt and water uptake in the land-crab, *Gecarcinus lateralis*. *Am. Zool.* **8**, 417-432.

Craig, D. H., Chapman, S. K. and Daff, S. (2002). Calmodulin activates electron transfer through neuronal nitric-oxide synthase reductase domain by releasing an NADPH-dependent conformational lock. *J. Biol. Chem.* **277**, 33987-33994.

Davies, S. A. (2000). Nitric oxide signalling in insects. *Insect Biochem. Molec. Biol.* **30**, 1123-1138.

Davies, S. A., Huesmann, G. R., Maddrell, S. H. P., O'Donnell, M. J., Skaer, N. J. V., Dow, J. A. T. and Tublitz, N. J. (1995). CAP(2b), a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **38**, R1321-R1326.

Davies, S. A., Stewart, E. J., Huesmann, G. R., Skaer, N. J. V., Maddrell, S. H. P., Tublitz, N. J. and Dow, J. A. T. (1997). Neuropeptide stimulation of the nitric oxide signaling pathway in *Drosophila melanogaster* Malpighian tubules. *Am. J. Physiol.* **42**, R823-R827.

Dow, J. A. T., Maddrell, S. H. P., Davies, S. A., Skaer, N. J. V. and Kaiser, K. (1994). A novel role for the nitric oxide cGMP signaling pathway: the control of epithelial function in *Drosophila*. *Am. J. Physiol.* **266**, R1716-R1719.

Erxleben, C. and Hermann, A. (2001). Nitric oxide augments voltage-activated calcium currents of Crustacea (*Idotea baltica*) skeletal muscle. *Neurosci. Lett.* **300**, 133-136.

Feelisch, M. and Martin, J. F. (1995). The early role of nitric-oxide in evolution. *Trends Ecol. Evol.* **10**, 496-499.

Ghosh, D. K. and Salerno, J. C. (2003). Nitric oxide synthases: domain structure and alignment in enzyme function and control. *Front. Biosci.* **8**, D193-D209.

Gibbs, S. M. and Truman, J. W. (1998). Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of *Drosophila*. *Neuron* **20**, 83-93.

Gunamalai, V., Kirubagaran, R. and Subramoniam, T. (2003). Sequestration of ecdysteroid hormone into the ovary of the mole crab, *Emerita asiatica* (Milne Edwards). *Curr. Sci.* **85**, 493-496.

Haase, A. and Bicker, G. (2003). Nitric oxide and cyclic nucleotides are regulators of neuronal migration in an insect embryo. *Development* **130**, 3977-3987.

Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L. and Marsden, P. A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J. Biol. Chem.* **269**, 33082-33090.

Hayashi, Y., Nishio, M., Naito, Y., Yokokura, H., Nimura, Y., Hidaka, H. and Watanabe, Y. (1999). Regulation of neuronal nitric-oxide synthase by calmodulin kinases. *J. Biol. Chem.* **274**, 20597-20602.

Hermann, A. and Erxleben, C. (2001). Nitric oxide activates voltage-dependent potassium currents of crustacean skeletal muscle. *Nitric Oxide* **5**, 361-369.

Imamura, M., Yang, J. and Yamakawa, M. (2002). cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, *Bombyx mori*. *Insect Molec. Biol.* **11**, 257-265.

Johansson, K. U. I. and Carlberg, M. (1994). NADPH-diaphorase histochemistry and nitric oxide synthase activity in deutocerebrum of the crayfish, *Pacifastacus leniusculus* (Crustacea, Decapoda). *Brain Res.* **649**, 36-42.

Johansson, K. U. I. and Mellon, D. (1998). Nitric oxide as a putative messenger molecule in the crayfish olfactory midbrain. *Brain Res.* **807**, 237-242.

Kean, L., Cazenave, W., Costes, L., Broderick, K. E., Graham, S., Pollock, V. P., Davies, S. A., Veenstra, J. A. and Dow, J. A. T. (2002). Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster*. *Am. J. Physiol.* **282**, R1297-R1307.

Komeima, K., Hayashi, Y., Naito, Y. and Watanabe, Y. (2000). Inhibition of neuronal nitric-oxide synthase by calcium/calmodulin-dependent protein kinase II alpha through Ser(847) phosphorylation in NG108-15 neuronal cells. *J. Biol. Chem.* **275**, 28139-28143.

Kone, B. C. (2001). Molecular biology of natriuretic peptides and nitric oxide synthases. *Cardiovasc. Res.* **51**, 429-441.

Kone, B. C., Kuncewicz, T., Zhang, W. Z. and Yu, Z. Y. (2003). Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am. J. Physiol.* **285**, F178-F190.

Korneev, S. A., Piper, M. R., Picot, J., Phillips, R., Korneeva, E. I. and O'Shea, M. (1998). Molecular characterization of NOS in a mollusc: expression in a giant modulatory neuron. *J. Neurobiol.* **35**, 65-76.

Kuzin, B., Regulski, M., Stasiv, Y., Scheinker, V., Tully, T. and Enikolopov, G. (2000). Nitric oxide interacts with the retinoblastoma pathway to control eye development in *Drosophila*. *Curr. Biol.* **10**, 459-462.

Kuzin, B., Roberts, I., Peunova, N. and Enikolopov, G. (1996). Nitric oxide regulates cell proliferation during *Drosophila* development. *Cell* **87**, 639-649.

Lachaise, A., Le Roux, A., Hubert, M. and Lafont, R. (1993). The molting gland of crustaceans: localization, activity, and endocrine control (a review). *J. Crust. Biol.* **13**, 198-234.

Lee, C. Y., Zou, H. S., Yau, S. M., Ju, Y. R. and Liau, C. S. (2000). Nitric

oxide synthase activity and immunoreactivity in the crayfish *Procambarus clarkii*. *Neuroreport* **11**, 1273-1276.

Luckhart, S. and Li, K. (2001). Transcriptional complexity of the *Anopheles stephensi* nitric oxide synthase gene. *Insect Biochem. Molec. Biol.* **31**, 249-256.

Luckhart, S. and Rosenberg, R. (1999). Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. *Gene* **232**, 25-34.

Luckhart, S., Vodovotz, Y., Cui, L. W. and Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc. Natl. Acad. Sci. USA* **95**, 5700-5705.

MacPherson, M. R., Pollock, V. P., Broderick, K. E., Kean, L., O'Connell, F. C., Dow, J. A. T. and Davies, S. A. (2001). Model organisms: new insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. *Am. J. Physiol.* **280**, C394-C407.

Mahadevan, A., Lappé, J., Rhyne, R. T., Cruz-Bermúdez, N. D., Marder, E. and Goy, M. F. (2004). Nitric oxide inhibits the rate and strength of cardiac contractions in the lobster *Homarus americanus* by acting on the cardiac ganglion. *J. Neurosci.* **24**, 2813-2824.

Maniere, G., Vanhems, E., Gautron, F. and Delbecque, J. P. (2003). Inhibition of ovarian steroidogenesis by cyclic GMP in a fly. *J. Endocrinol.* **177**, 35-44.

Morton, D. B. and Hudson, M. L. (2002). Cyclic GMP regulation and function in insects. *Adv. Insect Physiol.* **29**, 1-54.

Mungrue, I. N., Bredt, D. S., Stewart, D. J. and Husain, M. (2003). From molecules to mammals: what's NOS got to do with it? *Acta Physiol. Scand.* **179**, 123-135.

Nakane, M., Mitchell, J., Forstermann, U. and Murad, F. (1991). Phosphorylation by calcium calmodulin-dependent protein kinase-II and protein kinase C modulates the activity of nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **180**, 1396-1402.

Nathan, C. and Xie, Q. W. (1994). Nitric oxide synthases – roles, tolls, and controls. *Cell* **78**, 915-918.

Nighorn, A., Gibson, N. J., Rivers, D. M., Hildebrand, J. G. and Morton, D. B. (1998). The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. *J. Neurosci.* **18**, 7244-7255.

Nishida, C. R. and de Montellano, P. R. O. (2001). Control of electron transfer in nitric-oxide synthases. Swapping of autoinhibitory elements among nitric-oxide synthase isoforms. *J. Biol. Chem.* **276**, 20116-20124.

Regulski, M. and Tully, T. (1995). Molecular and biochemical characterization of dNOS – a *Drosophila* Ca^{2+} calmodulin-dependent nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **92**, 9072-9076.

Ribeiro, J. M. C. and Nussenzveig, R. H. (1993). Nitric oxide synthase activity from a hematophagous insect salivary gland. *FEBS Lett.* **330**, 165-168.

Roman, L. J., Martasek, P. and Masters, B. S. S. (2002). Intrinsic and extrinsic modulation of nitric oxide synthase activity. *Chem. Rev.* **102**, 1179-1189.

Rosay, P., Davies, S. A., Yu, Y., Sozen, M. A., Kaiser, K. and Dow, J. A. T. (1997). Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* **110**, 1683-1692.

Saïdi, B., De Bessé, N., Webster, S. G., Sedlmeier, D. and Lachaise, F. (1994). Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH) a neuropeptide which inhibits steroidogenesis in a crab. *Mol. Cell. Endocrinol.* **102**, 53-61.

Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M. E., Martasek, P., Roman, L. J., Masters, B. S. S., Jones, C. L. et al. (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J. Biol. Chem.* **272**, 29769-29777.

Schachtner, J., Homberg, U. and Truman, J. W. (1999). Regulation of cyclic GMP elevation in the developing antennal lobe of the sphinx moth, *Manduca sexta*. *J. Neurobiol.* **41**, 359-375.

Scholz, N. L. (1999). NO/cGMP signaling and the specification of motor networks in the crab stomatogastric ganglion. *Am. Zool.* **39**, 46a.

Scholz, N. L. (2001). NO/cGMP signaling and the flexible organization of motor behavior in crustaceans. *Am. Zool.* **41**, 292-303.

Scholz, N. L., Chang, E. S., Graubard, K. and Truman, J. W. (1998). The NO/cGMP pathway and the development of neural networks in postembryonic lobsters. *J. Neurobiol.* **34**, 208-226.

Scholz, N. L., de Vente, J., Truman, J. W. and Graubard, K. (2001). Neural network partitioning by NO and cGMP. *J. Neurosci.* **21**, 1610-1618.

Scholz, N. L., Labenia, J. S., De Vente, J., Graubard, K. and Goy, M. F. (2002). Expression of nitric oxide synthase and nitric oxide-sensitive guanylate cyclase in the crustacean cardiac ganglion. *J. Comp. Neurol.* **454**, 158-167.

Schuppe, H., Aonuma, H. and Newland, P. L. (2001a). Distribution of NADPH-diaphorase-positive ascending interneurones in the crayfish terminal abdominal ganglion. *Cell Tissue Res.* **305**, 135-146.

Schuppe, H., Aonuma, H. and Newland, P. L. (2001b). NADPH-diaphorase histochemistry in the terminal abdominal ganglion of the crayfish. *Cell Tissue Res.* **303**, 289-299.

Schuppe, H., Cuttle, M., Chad, J. E. and Newland, P. L. (2002). 4,5-Diaminofluoroscein imaging of nitric oxide synthesis in crayfish terminal ganglia. *J. Neurobiol.* **53**, 361-369.

Sedlmeier, D. and Fenrich, R. (1993). Regulation of ecdysteroid biosynthesis in crayfish Y-organs. I. Role of cyclic nucleotides. *J. Exp. Zool.* **265**, 448-453.

Seidel, C. and Bicker, G. (2002). Developmental expression of nitric oxide/cyclic GMP signaling pathways in the brain of the embryonic grasshopper. *Dev. Brain Res.* **138**, 71-79.

Skinner, D. M. (1985). Molting and regeneration. In *The Biology of Crustacea* (ed. D. E. Bliss and L. H. Mantel), pp. 43-146. New York, NY: Academic Press.

Spaziani, E., DeSantis, K., O'Rourke, B. D., Wang, W. L. and Weld, J. D. (1997). The clearance in vivo and metabolism of ecdysone and 3-dehydroecdysone in tissues of the crab *Cancer antennarius*. *J. Exp. Zool.* **279**, 609-619.

Spaziani, E., Mattson, M. P., Wang, W. N. L. and McDougall, H. E. (1999). Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. *Am. Zool.* **39**, 496-512.

Spaziani, E., Jegla, T. C., Wang, W. L., Booth, J. A., Connolly, S. M., Conrad, C. C., Dewall, M. J., Sarno, C. M., Stone, D. K. and Montgomery, R. (2001). Further studies on signaling pathways for ecdysteroidogenesis in crustacean Y-organs. *Am. Zool.* **41**, 418-429.

Stasiv, Y., Regulski, M., Kuzin, B., Tully, T. and Enikolopov, G. (2001). The *Drosophila* nitric oxide synthase gene (dNOS) encodes a family of proteins that can modulate NOS activity by acting as dominant negative regulators. *J. Biol. Chem.* **276**, 42241-42251.

Subramoniam, T. (2000). Crustacean ecdysteroids in reproduction and embryogenesis. *Comp. Biochem. Physiol. C* **125**, 135-156.

Suzuki, S., Yamasaki, K., Fujita, T., Mamiya, Y. and Sonobe, H. (1996). Ovarian and hemolymph ecdysteroids in the terrestrial isopod *Armadillidium vulgare* (Malacostracan Crustacea). *Gen. Comp. Endocrinol.* **104**, 129-138.

Talavera, E., Martinezlorenzana, G., Leonolea, M., Sanchezalvarez, M., Sanchezislas, E. and Pellicer, F. (1995). Histochemical distribution of NADPH-diaphorase in the cerebral ganglion of the crayfish *Cambarellus montezumae*. *Neurosci. Lett.* **187**, 177-180.

Taylor, H. H. and Taylor, E. W. (1992). Gills and lungs: the exchange of gases and ions. In *Microscopic Anatomy of Invertebrates, vol. 10, Decapod Crustacea* (ed. F. W. Harrison and A. G. Humes), pp. 203-293. New York, NY: John Wiley & Sons.

Torreilles, J. (2001). Nitric oxide: one of the more conserved and widespread signaling molecules. *Front. Biosci.* **6**, D1161-D1172.

Truman, J. W., DeVente, J. and Ball, E. E. (1996). Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects. *Development* **122**, 3949-3958.

Von Gliscynski, U. and Sedlmeier, D. (1993). Regulation of ecdysteroid biosynthesis in crayfish Y-organs: II. Role of cyclic nucleotide-dependent protein kinases. *J. Exp. Zool.* **265**, 454-458.

Wang, Y., Newton, D. C., Robb, G. B., Kau, C. L., Miller, T. L., Cheung, A. H., Hall, A. V., VanDamme, S., Wilcox, J. N. and Marsden, P. A. (1999). RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **96**, 12150-12155.

Warrier, S. R., Tirumalai, R. and Subramoniam, T. (2001). Occurrence of vertebrate steroids, estradiol 17 beta and progesterone in the reproducing females of the mud crab *Scylla serrata*. *Comp. Biochem. Physiol. A* **130**, 283-294.

Weiske, J. and Wiesner, A. (1999). Stimulation of NO synthase activity in the immune-competent lepidopteran *Estigmene acraea* hemocyte line. *Nitric Oxide Biol. Chem.* **3**, 123-131.

Zou, H. S., Chang, Y. Z., Chen, S. C., Yau, S. M., Shen, Y. L. and Lee, C. Y. (2002). Localization of NADPH-diaphorase and nitric oxide synthase activity in the eyestalk of the crayfish, *Procambarus clarkii*. *Zool. Studies* **41**, 244-250.