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Nitric Oxide Synthase Activity in the Molluscan CNS

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Abstract: Putative nitric oxide synthase (NOS) activity was assayed in molluscan CNS through histochemical localization of NADPH-diaphorase and through measurement of L-arginine/L-citrulline conversion. Several hundreds of NADPH-dependent diaphorase-positive neurons stained consistently darkly in the nervous system of the predatory opisthobranch *Pleurobranchaea californica,* whereas stained neurons were relatively sparse and/or light in the other opisthobranchs (*Philine, Aplysia, Tritonia, Flabellina, Cadlina, Armina, Coriphella,* and *Doriopsilla* sp.) and cephalopods (*Sepia* and *Rossia* sp.). L-Arginine/L-citrulline conversion was β-NADPH dependent, insensitive to removal of Ca^{2+}, inhibited by the calmodulin blocker trifluoperazine, and inhibited by the competitive NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) but not D-NAME. Inhibitors of arginase [L-valine and (+)-*S*-2-amino-5-iodoacetamidopentanoic acid)] did not affect L-citrulline production in the CNS. NOS activity was largely associated with the particulate fraction and appeared to be a novel, constitutive Ca^{2+}-independent isoform. Enzymatic conversion of L-arginine/L-citrulline in *Pleurobranchaea* and *Aplysia* CNS was 4.0 and 9.8%, respectively, of that of rat cerebellum. L-Citrulline formation in gill and muscle of *Pleurobranchaea* was not significant. The localization of relatively high NOS activity in neuron somata in the CNS of *Pleurobranchaea* is markedly different from the other opisthobranchs, all of which are grazers. Potentially, this is related to the animal's opportunistic predatory lifestyle. **Key Words:** Calcium—Calmodulin—Gastropoda—*Pleurobranchaea*—*Aplysia*—Cephalopoda.
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Nitric oxide (NO) may well act as a modulator of neuronal activity in molluscan nervous systems as it does in mammals (Moroz et al., 1993, 1995; Moroz and Gillette, 1995; Gelperin, 1994). Histochemical demonstration of NADPH-dependent diaphorase (NADPH-d) activity, a marker for NO synthase (NOS) (Bredt et al., 1991; Hope et al., 1991), in neurons of various invertebrates (Moroz et al., 1992; Elofsson et al., 1993) suggests that neuronal NOS activity is phyletically widespread. The presence of neuronal-like NOS in mollusks similar to that of mammals is supported by positive immunolabeling of neurons with antibodies against mammalian NOS (reviewed by Moroz et al., 1994). Thus, the growing body of evidence suggests that NO synthetic activity is present in molluscan nervous tissue and that NO has an important role in modulating neural discharges.

We screened histochemically for NADPH-d activity in the CNS and periphery in 11 genera of gastropods and cephalopods. We found that in most of the mollusks putative nitrergic neurons are relatively small and sparse. An exception was the CNS of the notaspid opisthobranch snail *Pleurobranchaea,* in which hundreds of neurons were labeled, including various identified and previously characterized cells. Pursuant to the investigation of the roles of NO in the molluscan nervous system, we undertook biochemical characterization of NOS activity by measuring the conversion of arginine to citrulline in nervous system homogenates of the *Pleurobranchaea* CNS and compared it with that of the opisthobranch *Aplysia* and that of the rat cerebellum.

MATERIALS AND METHODS

Animals

Specimens of *Pleurobranchaea californica* were obtained from SeaLife Supply (Sand City, CA, U.S.A.). *Aplysia californica* and *Flabellina iodinea* were obtained from Pacific Biomarine (Santa Monica, CA, U.S.A.). The opisthobranchs *Philine bakeri, Cadlina* sp., *Armina californica, Tritonia diomedia,* and *Coriphella sabulicula* and the cephalopod *Rossia pacifica* were trawled from Monterey Bay from depths of 20–50 m. The cephalopod *Sepia officinalis* was obtained from R. Hanlon (Marine Biomedical Institute, Galveston, TX, U.S.A.). The opisthobranch *Doriopsilla albopunctata* was collected from intertidal rocks in Pacific Grove, CA, U.S.A. Dissections were performed under cold anesthesia. Preparation of the rat cerebellum was done as described by Bredt and Snyder (1989).

NADPH-d histochemistry

The CNS and peripheral tissues were dissected and fixed in 4% paraformaldehyde in 0.4 *M* sodium phosphate buffer solution (pH 7.4) for 10–60 min at 4°C. NADPH-d staining and controls were performed on cryostat-cut sections and in whole-mount preparations, as described previously (Moroz et al., 1993), on the CNS of 18 *Pleurobranchaea* and 13

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Abbreviations used: AIPA, (+)-*S*-amino-5-iodoacetamidopentanoic acid; DTT, dithiothreitol; NADPH-d, NADPH-dependent diaphorase; NAME, *N*-nitroarginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; TFP, trifluoperazine.

Aplysia. For the other species three to six CNS preparations were analyzed.

NOS assay

CNS and samples of peripheral tissues were blotted on filter paper, rinsed in 50 m*M* HEPES buffer containing 1 m*M* EGTA (pH 7.1) on ice, reblotted, frozen, and stored at −80°C for subsequent analysis. NOS activity was analyzed according to the method of Bredt and Snyder (1989) with minor modifications (Ding et al., 1994). Ganglia were homogenized in ice-cold 50 m*M* HEPES/EDTA buffer and 1 m*M* dithiothreitol (DTT) at pH 7.1 (160 μl per CNS) and sonicated for 10 s. After homogenates were centrifuged at 10,000 *g* for 5 min, samples were passed through a Dowex 50 (Na^+ form) column to remove endogenous L-arginine. Cytosolic and microsomal fraction homogenates were separated in five pooled CNS by centrifugation at 100,000 *g* for 1 h at 4°C. The particulate fraction was washed with 1 m*M* KCl to remove loosely bound cytosolic proteins. L-[^{3}H]-Arginine (0.12 μ*M*, 35.7 Ci/mmol) and unlabeled L-arginine (0.3 μ*M*) were added to the homogenates, and the reaction was initiated by adding 0.5 m*M* β-NADPH, 10 μg/ml of calmodulin (Sigma), and 1.25 m*M* $CaCl_2$ in a total volume of 100 μl. After a 20-min incubation at room temperature, the reaction was stopped by addition of 900 μl of ice-cold 100 m*M* HEPES buffer containing 4 m*M* EDTA at pH 5.5. Reaction mixtures were passed through 1-ml Dowex 50 (Na^+ form) columns and washed with 2 ml of distilled water. The collected fractions containing L-[^{3}H]citrulline were counted in 4 ml of scintillation cocktail. The identity of the L-[^{3}H]arginine metabolite as L-[^{3}H]citrulline was confirmed using TLC. Samples of rat cerebellum were analyzed simultaneously in all experiments as a positive control. Total protein concentrations in the tissue extracts were determined by the assay of Bradford (1976). In some experiments the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; RBI) or its less active enantiomer D-NAME, the inhibitors of arginase, L-valine and (+)-*S*-2-amino-5-iodoacetamidopentanoic acid (AIPA; Alexis Co.), or the calmodulin inhibitor trifluoperazine (TFP) was added to the reaction mixture. In experiments analyzing the Ca^{2+} dependence of the arginine/citrulline conversion, incubation media contained 1 or 5 m*M* EGTA in HEPES buffer without added Ca^{2+}.

Data are mean ± SEM values. Each biochemical assay was repeated at least three times for a given tissue.

RESULTS

NADPH-d histochemistry

For all opisthobranchs, save *Pleurobranchaea,* ganglia showed relatively few NADPH-d-positive neurons: Generally <50 somata stained weakly to moderately, and fewer still (<10) stained blue to dark blue. Moreover, most labeled neurons in these animals were relatively small, <50 μm in diameter (data not shown). In the cephalopods' CNS, NADPH-d-positive somata were not found, and NADPH-d activity was observed in neuropil areas only, where it was relatively weak. These observations resemble those recently reported for *Sepia* (Chichery and Chichery, 1994). Intense NADPH-d labeling in central neuropil was observed only in *Aplysia* CNS. In contrast, NADPH-d activity darkly labeled up to 500 neuron somata in central ganglia of *Pleurobranchaea.* The majority of NADPH-d-positive neurons were in the pedal ganglia and ranged in size from 50 to 200 μm. Labeled somata were also found on the buccal ganglion

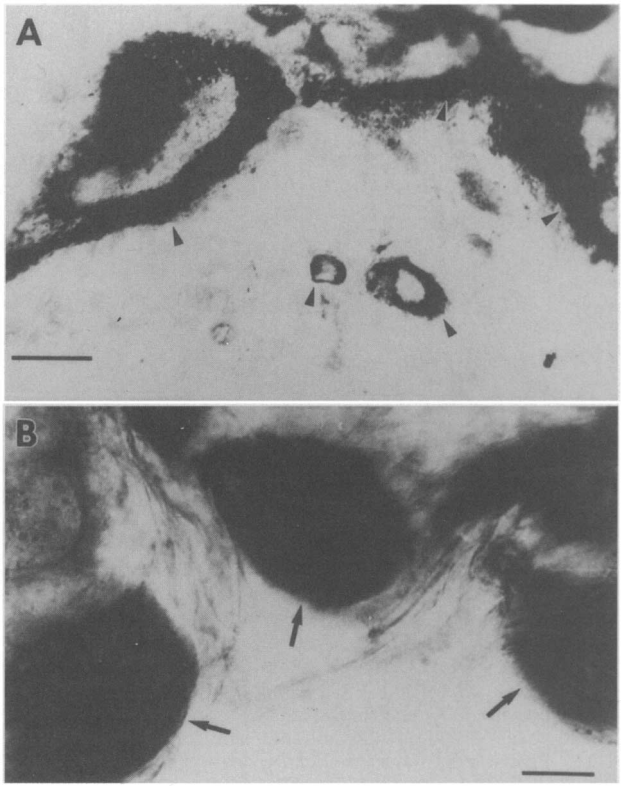

FIG. 1. NADPH-d activity as a marker of NOS activity in central neurons (arrows and arrowheads) of *Pleurobranchaea*. **A:** A section of the anterior cerebral lobe of the cerebropleural ganglion. **B:** Cell bodies in the posterior pleuroparietal lobe of the cerebropleural ganglion in a whole-mount preparation. Bars = 100 μm.

dorsal surface and in the cerebropleural ganglion, where soma diameters of the larger neurons were 150–500 μm (Fig. 1).

Arginine/citrulline conversion

Rates of L-[^{3}H]citrulline production in ganglion homogenates of *Pleurobranchaea* and *Aplysia* were 6.9 ± 0.4 and 16.8 ± 4.8 pmol/mg of protein/20 min, respectively. These values were 4.0 and 9.8% of that for rat cerebellum (170.6 ± 11.7 pmol/mg of protein/20 min). The time courses of L-[^{3}H]citrulline production was linear over 20 min for all three tissue types (data not shown).

L-[^{3}H]Citrulline production was very low in pedal muscles and gills of *Pleurobranchaea:* 5.8 ± 2.13 (n = 3) and 9.8 ± 3.1% (n = 3) of CNS activity, respectively. These values correspond to differences in NADPH-d staining, which was not observed in pedal muscle. In the gills several very small NADPH-d-positive cells were found, but their staining was very diffuse against a high background and was mimicked by glucose-6-phosphate dehydrogenase activity (authors' unpublished data).

Of the activity of L-citrulline production, 72 ± 4.9% was localized in the particulate fraction of *Pleurobranchaea* CNS homogenates. L-Citrulline production was NADPH dependent and was suppressed by TFP (Fig. 2A). Without added NADPH in the reaction, L-[^{3}H]citrulline production was reduced to <40%; it is unknown how much remaining activ-

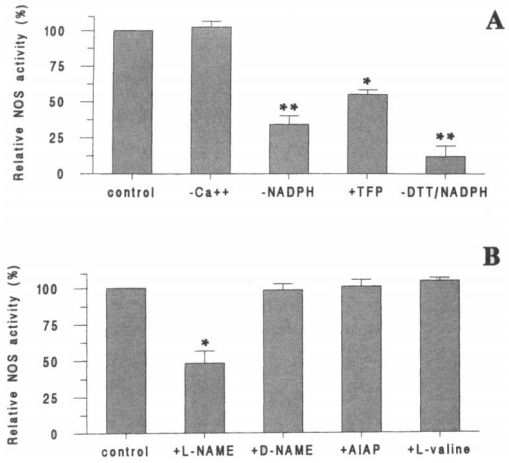

FIG. 2. L-[^{3}H]Arginine/L-[^{3}H]citrulline conversion in homogenates of the CNS of *Pleurobranchaea*. Values of relative NOS activity are expressed as percentages of the value obtained from the reaction mixture containing all cofactors. Enzyme activity was determined (**A**) in the absence of each cofactor indicated or in the presence of calmodulin antagonist or (**B**) in the presence of NOS or arginase antagonists: −Ca++, Ca^{2+}-free EGTA (1 m*M*)-containing reaction mixture; −NADPH, without β-NADPH in the reaction mixture; +TFP, in the presence of 80 μ*M* TFP; −DTT/NADPH, without both DTT and β-NADPH in homogenate and reaction mixture; +L-NAME, 20 μ*M* L-NAME; +D-NAME, 50 μ*M* D-NAME; +AIAP, 0.1 m*M* AIAP; and +L-valine, 20 m*M* L-valine. Data are mean ± SEM (bars) values of three to five experiments performed in triplicate. Asterisks indicate significant differences from control values: $^*p < 0.01$, $^{**}p < 0.001$ by Tukey–Kramer multiple comparisons test.

ity was due to endogenous NADPH. Omitting both NADPH and DTT reduced L-[^{3}H]citrulline production even further (Fig. 2A).

For the CNS of *Pleurobranchaea*, L-[^{3}H]citrulline production in media containing EGTA was not changed by omission of added Ca^{2+} (Fig. 2A), suggesting that L-[^{3}H]-citrulline production is calmodulin dependent but relatively independent of Ca^{2+}. Similar nonsensitivity of putative NOS activity to Ca^{2+} was observed for *Aplysia* nervous tissue ($n = 3$; data not shown). In contrast, similar Ca^{2+}-free conditions in reaction media for cerebellar tissue reduced L-[^{3}H]-citrulline production to 2.27 ± 1.17% of the control value, in accord with the known strong Ca^{2+} dependence of neuronal NOS in mammalian brain.

Addition of the NOS inhibitor L-NAME to extracts of *Pleurobranchaea* CNS significantly inhibited L-[^{3}H]-citrulline production; the less active enantiomer, D-NAME, was not effective under the same conditions (Fig. 2B). In homogenates not depleted of endogenous L-arginine on Dowex 50 columns, 1 m*M* L-NAME caused only 50% inhibition of citrulline production; however, only 20 μ*M* L-NAME induced similar inhibition after removal of endogenous L-arginine. L-[^{3}H]Citrulline production was not affected by either of the arginase blockers, L-valine or AIAP, which are reversible and irreversible arginase inhibitors, respectively (Fig. 2B).

DISCUSSION

L-[^{3}H]Arginine/L-[^{3}H]citrulline production potentially due to NOS activity was reported in the pond snail, *Lymnaea* (Elofsson et al., 1993), but a possible origin in arginase activity was not excluded, and the effects of NADPH, L(D)-NAME, Ca^{2+}, and calmodulin were not investigated. The present data are evidence that NOS is expressed in the CNS of molluscs and that it is particularly strongly expressed in *Pleurobranchaea* and *Aplysia*. We found fixative-resistant β-NADPH-d activity in all opisthobranchs examined; such activity in mammalian and insect nervous systems is associated with NOS (Hope et al., 1991; Bredt et al., 1991; Muller and Bicker, 1994). The NADPH-dependent conversion of arginine to citrulline in nervous system homogenates, its inhibition by the selective NOS inhibitor L-NAME but not by D-NAME, and the lack of effects of the urea cycle arginase inhibitors L-valine and AIAP are also consistent with actual NOS activity (reviewed by Knowles and Moncada, 1994). Moreover, work presently in progress in collaboration with S.-W. Norby using electron paramagnetic resonance to quantify NO directly is apparently confirming this interpretation.

The lack of Ca^{2+} dependence of molluscan NOS observed in our measurements from *Pleurobranchaea* and *Aplysia* contrasts with the strong Ca^{2+}/calmodulin dependence of NOS observed in arthropod CNS (Johansson and Carlberg, 1994; Muller and Bicker, 1994; Elphick et al., 1995) and with mammalian neuronal and endothelial forms of NOS (Knowles and Moncada, 1994). However, the susceptibility of enzyme activity to TFP suggests that molluscan NOS, like inducible forms of mammalian NOS (Knowles and Moncada, 1994), may constitutively bind calmodulin as a tightly held cofactor without Ca^{2+}.

A constitutive nature of *Pleurobranchaea* NOS is evident in the consistency of the histochemical results. The characteristics of *Pleurobranchaea* NOS activity thus suggest that the enzyme is an isoform that is novel in the combination of its neuronal origin, subcellular localization, sensitivity to calmodulin blockers, insensitivity to Ca^{2+}, and its constitutive nature. In invertebrates, other Ca^{2+}-independent constitutive isoforms of NOS so far known are nonneuronal, being found in hemocytes of the horseshoe crab (Radomski et al., 1991) and in insect Malpighian tubes (Choi et al., 1995). "Inducible" NOS activity, as opposed to constitutive, is induced by treatment with bacterial endotoxins, cytokines, and other intercellular signaling factors and has generally been found to be independent of Ca^{2+}/calmodulin (Knowles and Moncada, 1994). In mammals, some inducible isoforms of cerebellar granule neurons (Sato et al., 1995) and of nonneuronal tissues of mammals, including microglia (Wood et al., 1994) and hepatocytes (Evans et al., 1992; Iida et al., 1992), are Ca^{2+} independent but calmodulin dependent, as may be the case for constitutive NOS of *Pleurobranchaea*.

It is also of interest to find that NOS activity in the molluscan CNS can be around 10% of the value for rat cerebellar tissue. NOS activity in cerebellar tissue is two to eight times higher than in other areas of the rat brain (Bredt et al., 1991; Lin et al., 1994); thus, activity of molluscan NOS may be quite comparable to that of the rat CNS as a whole, under the specific conditions in which we measured it.

The biochemical measures are also consistent with observations from the NADPH-d histochemistry. *Pleurobranchaea* differs from the other opisthobranchs examined here in its predatory lifestyle and in its neurophysiological adaptations that support a habit of opportunistic gluttony (Gillette et al., 1980; Gillette and Gillette, 1983). The high levels of somatic neuronal NADPH-d activity observed are also

unique to *Pleurobranchaea,* and we speculate that they could somehow contribute importantly to the modulation of neural network activity underlying the foraging activity of *Pleurobranchaea* (Moroz et al., 1995).

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