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Nitric Oxide Synthase Imunolabeling in the Molluscan CNS and Peripheral Tissues

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NOS immunoreactivity was assayed in CNS and peripheral tissues of the sea slugs *Pleurobranchaea californica, Tritonia diomedea* and *Aplysia californica* using different antisera against mammalian nitric oxide synthase in Western blots. Polyclonal anti-nNOS labeled at 250, 185, 170, 155, 100, 75, and 65 kD in extracts of *Pleurobranchaea* CNS, salivary gland and esophagus but not of gills or muscle. The labeling pattern for *Tritonia* in bands at 250, 200, 120/110, 100, 69, 65, and 60 kD differed somewhat. Anti-nNOS labeling in *Aplysia* was markedly different, with bands labeled only at 69 and 60 kD in CNS extracts, and at 200, 190, 69 and 60 kD in salivary and esophagus extracts. The wide variation in NOS immunoreactivity is consistent with species differences in tissue localization and biochemical properties of molluscan NOS isoforms.

Key Words: **neuronal NOS; inducible NOS; endothelial NOS; *Pleurobranchaea; Aplysia; Tritonia;* gastropoda.**

Nitric oxide (NO) modulates multiple aspects of synaptic transmission and neuron excitability in invertebrate nervous systems (1, 2, 3). Presently, the tissue distributions and range of properties of nitric oxide synthase (NOS) and its isoforms are in early stages of investigation in invertebrates, and results from diverse laboratories suggest substantial variability may exist. For arthropods, a Ca^{2+}-dependent constitutive NOS resembling the neuronal NOS of mammals has been demonstrated in the CNS of crayfishes (4) and insects (5, 6). Coelenterates also have a neuronal type NOS whose activity is substantially Ca^{2+}-dependent (7). In molluscs, NOS activity has been measured in CNS of the opisthobranchs *Aplysia* and *Pleurobranchaea* (8), and of the pulmonates *Lymnaea* (9, 10) and *Helix* (11). Measured in terms of arginine to citrulline conversion (substrate and coproduct, respectively) in homogenate extracts, NOS activity in the opisthobranchs was substantial but not Ca^{2+} dependent (8). In contrast, NOS activity measured in affinity purified extracts of *Lymnaea* CNS was decreased by at least half in Ca^{2+}-free saline (10), while NOS activity from *Helix* ganglia similarly assayed was reduced by 90% by Ca^{2+} removal (11). While these results might reflect in part the different methods of extract preparations, they also suggest that the character of NOS in the CNS may vary across species.

As a part of a comparative analysis of NOS in molluscs, we have characterized NOS immunoreactivity in CNS and peripheral tissues of three marine opisthobranchs commonly used in CNS research; the notaspid *Pleurobranchaea californica,* the nudibranch *Tritonia diomedea,* and the cephalospidean *Aplysia californica.* In Western blot assays of tissue extracts using antisera against the three mammalian NOS isoforms: neuronal NOS (nNOS, type I), endothelial NOS (eNOS, type III) (12), and the inducible NOS (iNOS; type II), we found wide variations among both species and tissues in antigen staining, antigen molecular weight and relative levels of NOS antigenicity, as described below.

MATERIALS AND METHODS

Specimens of *P. californica* MacFarland, 1966 (*Opisthobranchia: Notaspidea,* n = 11); and *T. diomedea* Bergh, 1894 (*Opisthobranchia: Nudibranchia,* n = 4) and *A. californica* Cooper, 1863 (*Opisthobranchia: Anaspidea,* n = 5) were collected from the Pacific coast of California and maintained in artificial sea water for up to a month before experiments. CNS and peripheral tissues were dissected, blotted on filter paper, weighed and quickly frozen on dry ice. All samples were kept at −70°C for up to 36 hours until use.

Protein extracts were prepared from frozen tissue by addition of enough 0.1% SDS to make samples approximately 20 mg protein/ml. Samples were kept on ice, homogenized by grinding and sonicated (3 × 30 sec). Insoluble material was removed by brief centrifugation (14,000 rpm, 1 min). Protein concentration of each sample was determined by Bradford assay prior to adding an equal volumes of 2× denaturing buffer (0.125 M Tris-HCl, pH 6.8, 1% SDS, 30% glyc-

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Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; CNS, central nervous system.

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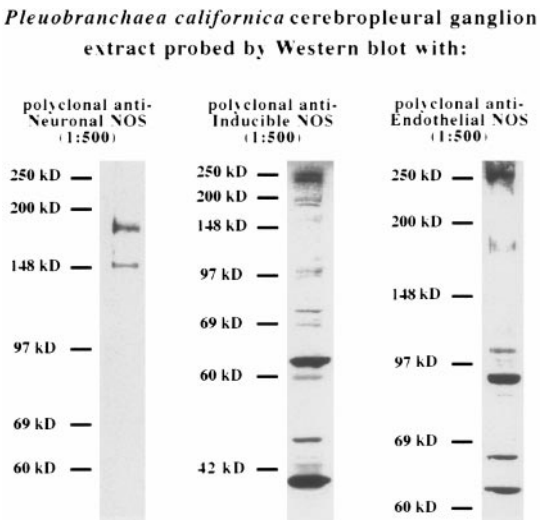

FIG. 1. NOS immunoreactivity in the CNS of *Pleurobranchaea californica* probed with polyclonal antisera against three mammalian NOS isoforms.

erol, 5% 2-mercaptethanol, 0.1% bromphenol blue). Samples were boiled (3 min) and subjected to standard SDS-PAGE electrophoresis. 10 μg protein were loaded into each lane. Gels were transferred to nitrocellulose by standard methods. Parallel gels were run and subjected to silver staining (Bio-Rad) to assess relative protein loading. Nitrocellulose blots were probed with peptide-derived antibodies from Transduction Labs, including polyclonal rabbit anti-nNOS (1:500); polyclonal rabbit anti-eNOS (1:500); polyclonal rabbit anti-iNOS (1:500); monoclonal mouse anti-nNOS (1:2500); monoclonal mouse anti-eNOS (1:2500); and, monoclonal mouse anti-iNOS (1:2500). Additionally, a rabbit polyclonal antibody against the entire nNOS protein (1:1000) was tested (13). Immunoreactivity was visualized using horseradish peroxidase-linked goat anti-rabbit (Chemicon) or goat anti-mouse (Zymed) secondary antibody and ECL fluorescence detection system (Amersham). Specificity of immunoreactive bands was determined by pre-absorbing nNOS antibodies with 25 g/ml nNOS purified from baculovirus-infected insect cells (14) for 60 min at room temperature before probing nitrocellulose blot.

Rat cerebellum, pituitary tissue, human endothelial cells and mouse macrophage extracts were used as appropriate positive controls (15) and run simultaneously with molluscan tissues in each experiment. The pedal muscles, buccal muscles and esophagus from *Pleurobranchaea* were used as negative controls, since our earlier measures of specifically fixative resistant NADPH-diaphorase reactivity (a marker for NOS) and detectable NOS activity in L-arginine/L-citrulline conversion were negative (8, 16).

RESULTS

NOS immunolabeling in Pleurobranchaea. We compared monoclonal (3 peptide-derived) and polyclonal (3 peptide-derived; 1 against the entire protein) antisera specific to each of the NOS isoforms against extracts from CNS by Western analysis. In all experiments, polyclonal antisera against mammalian NOS isoforms were more effective than monoclonal antisera at labeling specific protein bands of molluscan tissue extracts. Monoclonal antisera did label many of the same bands on blots as the respective polyclonal antisera, however, to a much lower intensity. The most intense specific labeling was observed using neuronal anti-NOS polyclonal antisera (Fig. 1). Both anti-nNOS antisera produced similar results; however, the peptide-derived polyclonal antisera identified fewer bands at a higher intensity than other antisera used. CNS samples exhibited strong immunoreactivity mainly in ≥250 and 155 kD bands. Among the peripheral tissues, the 155 kD band was found only in the gill. Muscle showed immunoreactivity at relatively low molecular weights (≤110/100 kD), whereas both high (≥250, 185, 170) and low molecular weight nNOS immunoreactive proteins (≤100 kD) were observed in salivary gland and the esophagus.

In all tissues tested, the 155, 110, and 100 kD bands disappeared when nNOS antisera was pre-absorbed with bacterially-expressed nNOS (data not shown). Blots also indicated crossreactivity with lower molecular weight proteins (≤75 kD) which displayed no appreciable loss of immunoreactivity during pre-absorption experiments. Binding of nNOS antisera to lower molecular weight proteins has been demonstrated previously (15). These bands may represent related proteins or breakdown products of full length NOS proteins. Possibly, pre-absorption may have been insufficient to fully deplete primary nNOS antisera of antibodies which cross-react with these species.

Both eNOS and iNOS antisera labeled several proteins in the CNS with different molecular masses compared to nNOS immunolabeling (Table 1, Fig. 1). Antisera to iNOS recognized a large number of bands ranging from 40 to 250 kD. Most of these bands were considered non-specific as they were not lost when antibody was pre-absorbed with a mammalian NOS. Similar patterns were also observed with eNOS antisera, but the labeling was slightly less intense.

TABLE 1

A Comparison of the Principal Immunoreactive Bands in the CNS of Three Molluscan Species Using Anti-NOS Peptide-Derived Polyclonal Antisera

Approximate molecular weight of cross-reactive bands (kD)				
Pleurobranchaea californica			*Aplysia californica*	*Tritonia diomedea*
nNOS	eNOS	iNOS	(nNOS)	(nNOS)
≥250	≥250	≥250	—	250
		190	—	200
	185	185	—	—
155			—	—
	110	100	—	130, 120, 110, 100
	90		85	85
	67, 63	65	69, 63, 60	70–60
		50	—	—
		40	—	–

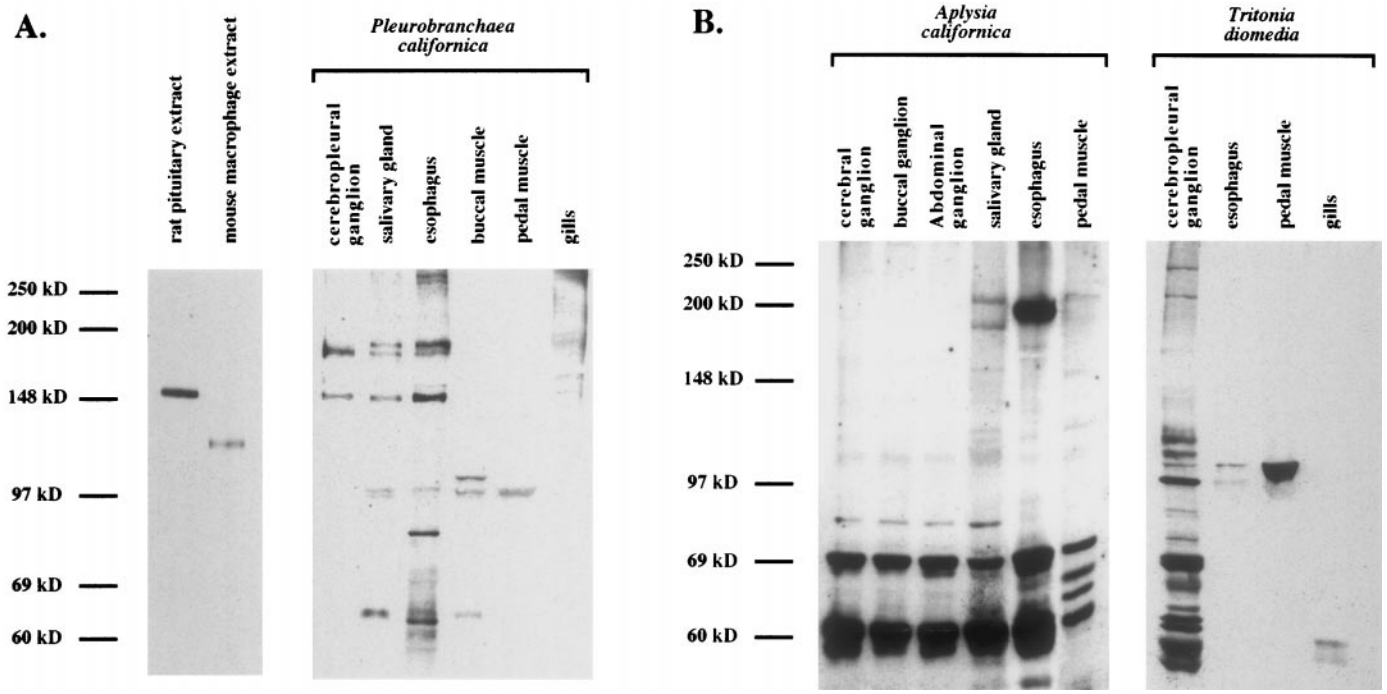

FIG. 2. A comparison of NOS immunoreactive bands in peripheral tissues from different molluscan species using peptide-derived polyclonal antisera. Rat pituitary and mouse macrophage extracts were used as controls for mammalian nNOS and iNOS, respectively. (A) Extracts from *Pleurobranchaea.* (B) Extracts from *Aplysia* and *Tritonia.*

NOS immunolabeling in Aplysia and Tritonia. Patterns of nNOS-immunolabeling in the CNS of *Aplysia* differed markedly from *Pleurobranchaea* (Table 1, Fig. 2); *Tritonia* also differed in labeling pattern, but less so than *Aplysia.* None of the NOS-immunoreactive bands in *Aplysia* corresponded to labeled *Pleurobranchaea* proteins. Blots of *Aplysia* CNS samples displayed only 69 and 60 kD bands, whereas 200, 190 and 69, 60 kD bands were found in salivary and esophagus extracts. In *Tritonia* CNS extracts, anti-nNOS antiserum labeled proteins at 250, 200, 120/110, 100, 69, 65 and 60 kD. In peripheral tissues only 110/100 and 60 kD proteins were observed.

DISCUSSION

Selectivity of the anti-NOS antisera has been previously tested in a range of mammalian tissues. In mammals nNOS is a 155 kD protein whose labeling is selectively out-competed in pre-absorption experiments. Labeling of low molecular weight proteins (around 65 kD) in rat cerebellum and pituitary tissues as well as in molluscan preparations was not affected by pre-absorption experiments, suggesting that their labeling was not specifically related to NOS immunoreactivity. Labeling of a 155 kD (133 kD) protein in *Pleurobranchaea* CNS was quite specific, since labeling was selectively removed in preadsorbtion experiments. This protein was detected only in the CNS, corresponding to the specific tissue localization of NOS activity in biochemical measures (15). Thus, this protein is a likely candidate for a molluscan form of neuronal NOS. In the insect *Drosophila* the estimated molecular weight for NOS is in the same range, 130-150 kD (17, 18), whereas a higher MW has been reported for nNOS from *Lymnaea* (10).

Comparison of the observed immunolabeling in *Pleurobranchaea* with the previously described distribution of NADPH diaphorase activity, a histochemical marker for NOS, showed appreciable similarity with one notable difference. That is, significant NADPH diaphorase activity was observed in central neurons, salivary gland and gills but was only weak or non-detectable in peripheral structures (16); however, intense NOS immunolabeling was found in *Pleurobranchaea* esophagus at 250, 185, and ~150 kD. Potentially, the immunoreactivity of the esophagus represents inactive enzyme in this tissue. In salivary gland, intense NADPH-d labeling coincided with intense NOS immunolabeling of bands at >250, 190 and 185 kD, but not at 155 kD (Table 2).

It is of substantial interest that such different patterns of nNOS immunoreactivity were observed in the opisthobranchs *Aplysia, Tritonia* and *Pleurobranchaea,* which also markedly differed in their tissue patterns of NADPH-d reactivity. Immunolabeled high molecular weight proteins were noted from multiple tissues of both *Aplysia* and *Tritonia,* but the 155 kD

TABLE 2

A Comparison of the Principal Immunoreactive Bands in Peripheral Tissues from Three Molluscan Species Using Peptide-Derived Polyclonal NOS Antisera

	Approximate molecular weight of cross-reactive bands (kD)		
Species: Tissue	*Pleurobranchaea californica* (nNOS)	*Aplysia californica* (nNOS)	*Tritonia diomedea* (nNOS)
Buccal muscles	110, 100	NT	NT
	65		
Pedal muscles	—	200	—
	100	—	110
	—	69, 67, 65, 63	—
Esophagus	≥250	—	—
	190	190	—
	185	—	—
	100	—	110, 100
	75	—	—
	65	—	—
Gills	≥250	NT	—
	190–185		—
	155		—
	—		60
Salivary glands	≥250	200	NT
	190	—	
	185	180	
	100	—	
	65	69, 63, 60	

protein found in *Pleurobranchaea* was not detected. In molluscs, NOS activity is broadly manifested in tissues and central neurons involved in food handling and feeding behavior, including salivary glands, esophagus, chemosensory epithelia, and specific CNS elements (19, 20, 21, 22). *Aplysia*, *Tritonia* and *Pleurobranchaea* vary broadly in their feeding ecology, being respectively a grazing herbivore, a grazing sessile cnidarian specialist, and an opportunistic general predator. These correlations suggest to us that the marked differences in tissue localization and molecular weights of NOS immunoreactivity may be related to differences in the role of NOS in food-handling. We suggest that such differences may have been involved in broad evolutionary changes in the adaptive radiation and properties of NOS isoforms among the gastropod molluscs.

These results suggest the presence of NOS isoforms that may vary broadly both among tissues and across molluscan species. We hope that ongoing efforts to characterize NOS activity in diverse tissues and species in our's and others' laboratories will provide useful information that can further physiological studies of the regulatory actions of NO and its synthetic enzyme in molluscan model systems.

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