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Nitric oxide (NO) synthase immunoreactivity in the starfish *Marthasterias glacialis*

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Abstract. The neuroendocrine system of the starfish *Marthasterias glacialis* was investigated immunocytochemically using antisera specific for rat neuronal, bovine aortic endothelial, and mouse macrophage, nitric oxide (NO) synthases. Immunoreactivity was detected only with the antibodies specific for the neural enzyme, in the ectoneural and hyponeural tissues of the radial nerve cords and in the basiepithelial plexus and endocrine cells of the digestive tract. The pyloric stomach showed more immunoreactive structures than the other digestive organs, with the rectal caeca showing the least activity. Immunoreactive endocrine cells were located in the cardiac and pyloric stomachs and in the pyloric caeca. Co-localization of the enzyme immunoreactivity, and the staining for NADPH-diaphorase, demonstrate the presence of NO synthase in echinoderms. These results provide further evidence that NO is a neuronal messenger of early phylogenetic origin which has been conserved throughout evolution.

Key words: Nitric oxide synthase – Nervous system – Gut – Endocrine cells – Immunocytochemistry – *Marthasterias glacialis* (Echinodermata)

Introduction

Recent studies have demonstrated that the neuroendocrine system of echinoderms possesses numerous regulatory substances, including classical neurotransmitters, peptides and hormones (Pentreath and Cottrell 1968; Cottrell and Pentreath 1970; Huet and Franquinet 1981; Bisgrove and Burke 1986; Nakajima 1988; Shirai et al. 1987; Mladenov et al. 1989; García-Arrarás et al. 1991; Welsch et al. 1989; Elphick et al. 1989; Elphick et al. 1991; Martínez et al. 1993).

The L-arginine: nitric oxide (NO) pathway is a newly discovered regulatory system involved in a variety of biological functions (Moncada 1992). Nitric oxide is synthesized from L-arginine by a group of enzymes, the NO synthases (Moncada et al. 1991). Nitric oxide synthases require NADPH as a co-factor and are inhibited by some analogues of L-arginine. At present two broad types of enzymes have been identified, i.e. constitutive and inducible (Moncada et al. 1991; Forstermann et al. 1991). Constitutive NO synthases are present in endothelium, brain, adrenal glands and platelets (Moncada et al. 1991). The inducible enzyme, which is synthesized in response to cytokines and inflammatory mediators, has been identified in macrophages, hepatocytes, vascular smooth muscle, endothelial cells (Moncada et al. 1991) and neutrophils (Rimele et al. 1991).

Nitric oxide synthases were originally identified in mammalian tissues. More recently, however, they have been found in the haemocytes of the arthropod *Limulus polyphemus* (Radomski et al. 1991), in several ganglia of the snail *Lymnea stagnalis* (Elphick et al. 1993a) and in the cerebral ganglion of the locust *Schistocerca gregaria* (Elphick et al. 1993b). Furthermore, an antibody raised against mammalian NO synthase has been shown to cross-react with amphibian tissues (Li et al. 1992). We have now used specific antisera against mammalian isoforms of the NO synthase to investigate the presence, nature and distribution of this enzyme in echinoderms.

Materials and methods

Three adult specimens of the starfish *Marthasterias glacialis* were collected from the Cantabrian sea (Spain). After anaesthesia with magnesium chloride, a careful dissection was performed. Pieces of radial nerve cord, cardiac and pyloric stomachs, pyloric caeca, intestine and rectal caeca were fixed in isotonic GPA (glutaraldehyde, picric and acetic acids), dehydrated and embedded in paraffin.

Tissues were sectioned at a thickness of 3 μm and immunocytochemistry using the avidin-biotin-horseradish peroxidase complex (Hsu et al. 1981), was performed. After removal of paraffin, intrinsic peroxidase was blocked by treatment of the sections for 30 min with a solution of methyl alcohol containing 3% H_2O_2. Background blocking was performed by incubating with normal swine serum diluted 1:20 for 30 min prior to the incubation with specific antisera (see below). After incubation with the primary antibody the sections were washed in TRIS-buffered saline (TBS 0.05 M, pH 7.36, 0.55 M

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NaCl), and subsequently incubated for 30 min either in biotinylated swine antirabbit IgG (Dakopatts E353, Glostrup, Denmark) for the polyclonal antisera, or in biotinylated rabbit antimouse IgG (Dakopatts E354) for the monoclonal, both at a dilution of 1:200. After rinsing as above, the sections were further incubated with the avidin-biotinylated peroxidase complex (1:100 dilution, Dakopatts K355) for 30 min. After a final rinse, peroxidase activity was visualized using diaminobenzidine (50 mg per 100 ml) and H_2O_2 (60 µl per 100 ml). The sections were lightly counterstained with Harris's hematoxylin.

Antisera

Four specific antisera against different isoforms of the NO synthase were used in this study. All were incubated at 4°C for 16–20 h at the specified dilutions:

1. Rabbit serum against purified rat brain NO synthase, characterized as described by Springall et al. (1992) and diluted 1:1000.
2. From the deduced sequence of the cloned rat neural NO synthase (Bredt et al. 1991), the amino acid sequence LPLLQANGND-PELFQIPPEL was selected as a possible exposed region on the native enzyme, as described by Riveros-Moreno et al. (1993). The peptide was synthesized with an added cysteine at the carboxy terminal to allow an ordered binding to the carrier protein Keyhole Limpet Haemocyanin (KLH). The conjugated peptide was used to raise antibodies in rabbits. The immune serum was used at a dilution of 1:1000.
3. The deduced sequence of the cloned murine inducible NO synthase (Lyons et al. 1992) was analyzed in a similar fashion to the brain enzyme. An amino acid sequence with low homology to the constitutive enzyme was selected and synthesized; QNGSPQLLTGTAQNVPESLDKLHVT. A carboxy terminal cysteine was added for binding to KLH as the carrier protein and the peptide was injected into rabbits. The specificity of the antiserum raised has been described in Hamid et al. (1993). The serum was used at a dilution of 1:750.
4. Monoclonal antibody for the constitutive endothelial isoform of the bovine NO synthase, as described by Pollock et al. (1993), was used at a dilution of 1:1000.

Controls

Mammalian tissues (digestive tract) were used as positive controls and substitution of the specific antibody by a non-immune serum was used as a negative control. The specificities of the antisera were confirmed by pre-absorption of the sera with the corresponding antigens and with the conjugated carrier-peptide for the synthetic peptides.

NADPH-diaphorase staining

Starfish tissues were dissected out, small pieces were immersed in phosphate-buffered saline (PBS, pH 7.4) containing 20% sucrose and embedded in OCT compound (Miles 4583, Elkhan, USA). Cryostat sections (5 µm thick) were incubated in PBS containing 1.2 mM β-NADPH (Fluka 93220, Switzerland) and 0.3 mM nitro blue tetrazolium (Boehringer 1175041, Mannheim, Germany) for 10–30 min at 37°C. After a thorough rinse in PBS the sections were mounted in PBS-glycerol (1:1).

Results

Immunoreactivity for NO synthase was found with 2 antisera raised against the neural constitutive form of the enzyme, while no immunostaining was observed using the antibodies against the endothelial or the inducible forms. Both antibodies raised against the mammalian neural form yielded similar results, although the immunoreactivity observed with the antibody raised against the synthetic peptide was stronger. Hence the results and figures shown are those using this antiserum.

The 5 radial nerve cords, together with the circumoral nerve ring, are the major components of the starfish's central nervous system. These nerve tracts extend along the ventral surface of each arm and contain two distinct parts (Fig. 1), the thick ectoneural system on the oral side and the aboral hyponeural system that lies above the ectoneural tissue. The two parts are separated by a thin basement membrane.

Nitric oxide synthase immunoreactivity was detected in both the ectoneural and the hyponeural systems (Fig. 1). Some ectoneural (Fig. 1B) and hyponeural cell bodies (Fig. 1C), a few beaded fibres in the axonal region of the ectoneural tissue, and some nerves in the neighbourhood of the cuticular surface (Fig. 1A) were positive.

The innervation of the alimentary tract of the starfish consists of two nervous plexuses, separated by a connective layer; these comprise a basiepithelial plexus running among the basal processes of the digestive epithelium and a subcoelomic plexus innervating the muscle cells in the coelomic face (Fig. 2). Immunoreactivity to NO synthase was restricted to the luminal side of the digestive wall, where epithelial endocrine cells and nerves of the basiepithelial plexus (Fig. 2) were stained. There were no differences between the mucosecretory and the current-producing regions of the cardiac stomach but in pyloric caeca the staining in the current zones (Fig. 3) was stronger than in the pouches. The pyloric stomach displayed more immunoreactive structures than the other organs, showing a thick NO synthase-positive basiepithelial plexus and numerous endocrine cells (Fig. 4). The same plexus in the intestine showed a discrete reactivity (Fig. 5) and the basiepithelial plexus of the rectal caeca presented the weakest NO synthase immunoreactivity (Fig. 6). Positive endocrine cells were found in both stomachs and in the pyloric caeca (Figs. 2–4) but none were observed in the intestine or the rectal caeca.

Fig. 1. A Longitudinal section of radial nerve cord showing some NO synthase-positive hyponeural cells (*small arrows*), and few immunoreactive fibres in ectoneural tissue (*arrow*) and below the cuticle (*arrowheads*). **B** Detail of small positive neuronal bodies in the ectoneural tissue. **C** Positive hyponeural cells. **A** × 375. *Bar*: 20 µm. **B** and **C** × 600. *Bar*: 20 µm

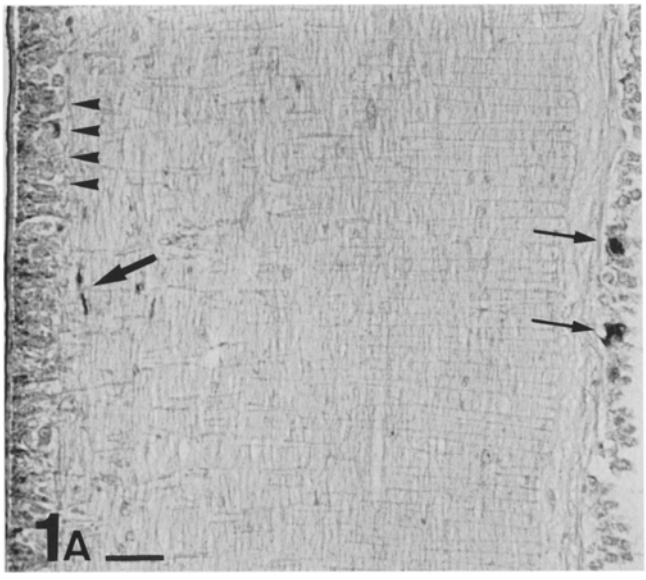
Fig. 2. Transverse section of the mucosecretory region of cardiac stomach. The basiepithelial plexus (*arrowheads*) and an endocrine cell are clearly immunoreactive. The subcoelomic plexus (*arrows*) is not stained. × 600. *Bar*: 20 µm

Fig. 3. Current zone of a pyloric caecum. A slender epithelial endocrine cell that probably contacts the basiepithelial plexus shows a strong immunoreactivity. × 600. *Bar*: 20 µm

Fig. 4. Panoramic view of the pyloric stomach. NO synthase immunoreactivity can be observed in the broad basiepithelial plexus (*arrowheads*) and in endocrine cells (*arrows*). *Inset*: Detail of the endocrine cells. × 150. *Bar*: 50 µm. *Inset* × 600. *Bar*: 20 µm

Fig. 5. Transverse section of the intestine. The basiepithelial plexus is clearly stained. × 375. *Bar*: 20 µm

Fig. 6. Rectal caecum. A faint immunoreactivity can be observed in the basiepithelial plexus. × 150. *Bar*: 50 µm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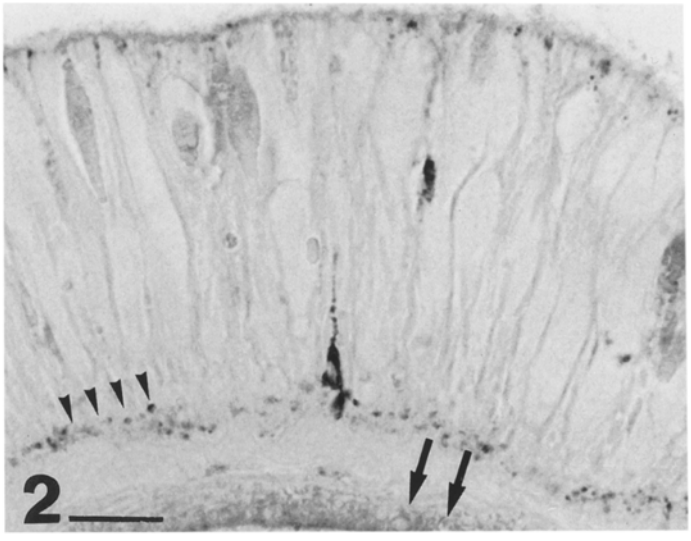
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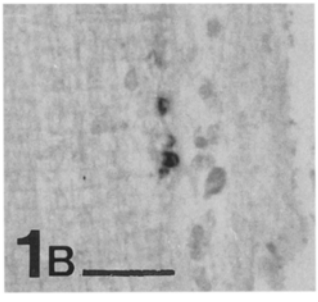
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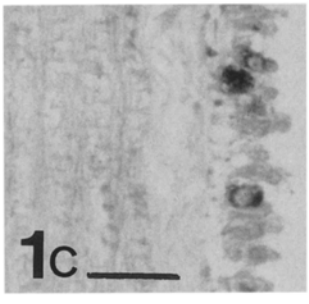
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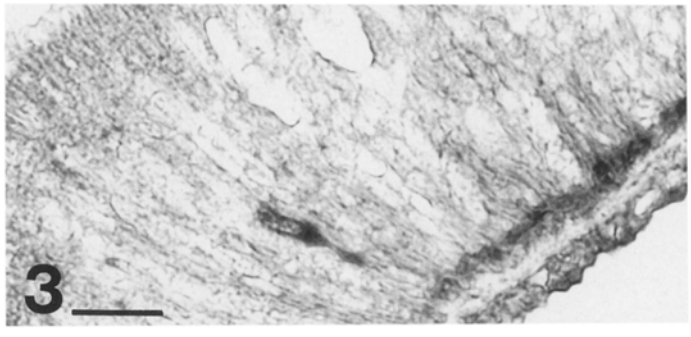
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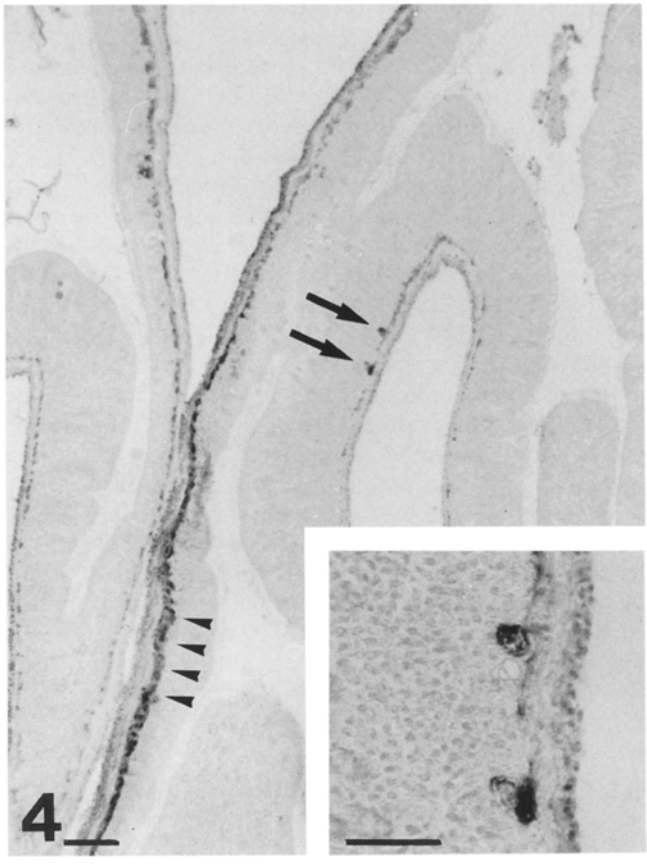
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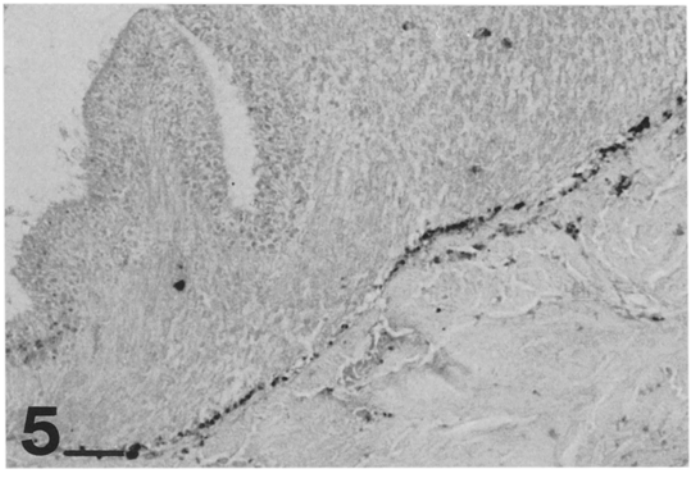
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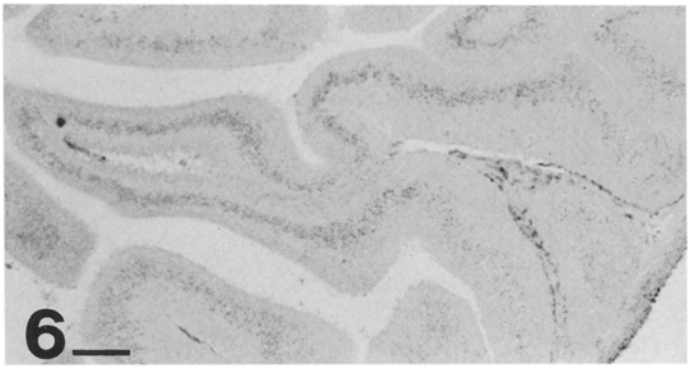
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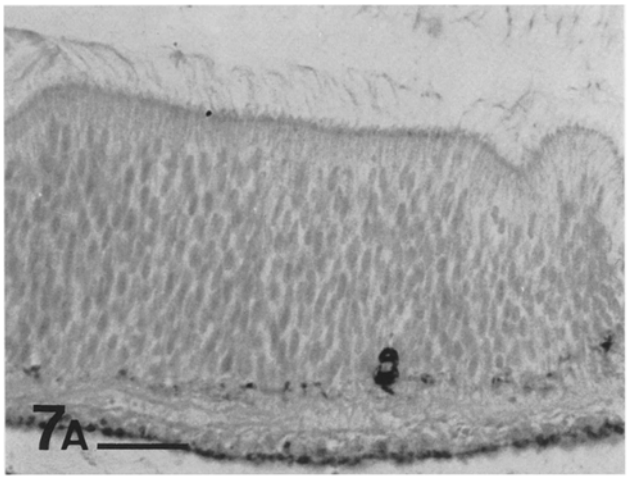

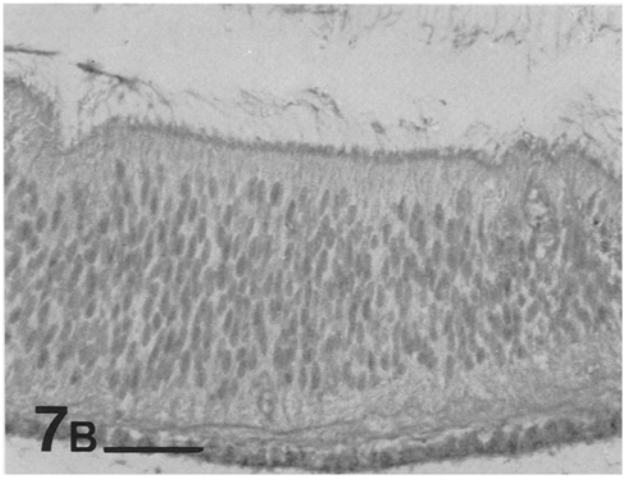

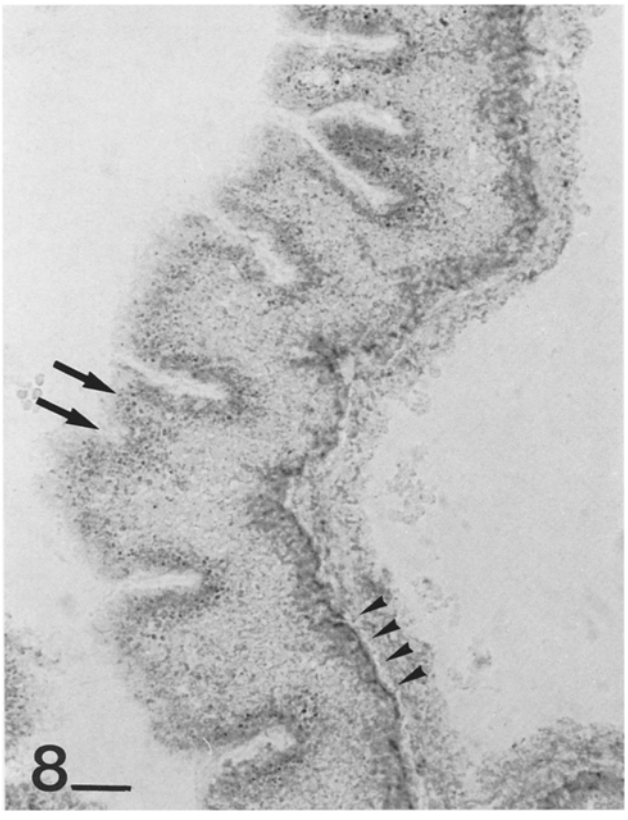

Absorption controls demonstrated the specificity of the antisera. The staining was abolished after incubation with the synthetic peptide (Fig 7) or the rat brain extract. Pre-incubation with KLH did not alter the immunoreactivity. The reaction product of the NADPH-diaphorase staining was located in the same areas that displayed NO synthase immunoreactivity, as well as in the apical region of the cells (Fig. 8).

Discussion

Our results demonstrate the presence of a NO synthase in the neuroendocrine system of an echinoderm, the starfish *Marthasterias glacialis*. This enzyme is constitutive, and reacted positively with an antibody raised against a synthetic peptide belonging to the sequence of the neuronal constitutive mammalian NO synthase, and to an antibody raised against the whole enzyme. In addition, this immunoreactivity co-localized with regions positive for NADPH-diaphorase which in the mammalian nervous tissue correspond to the NO synthase (Hope et al. 1991). Furthermore, there was no immunoreactivity with an antibody raised to an amino acid sequence belonging to the inducible macrophage enzyme, which has a low amino acid identity ($\sim$ 12%) with the constitutive enzyme and no reactivity with a monoclonal antibody specific for the endothelial form of the enzyme.

All this suggests that the detected enzyme is similar to the mammalian constitutive neuronal NO synthase (Bredt et al. 1990) and that this enzyme has therefore been largely conserved throughout evolution. This, together with the finding of NO synthase in amphibians (Li et al. 1992), molluscs (Elphick et al. 1993a) and arthropods (Elphick et al. 1993b), also indicates a widespread physiological involvement of NO across different phyla.

The role of NO in echinoderm biology requires further investigation. However, it is interesting that the most abundant immunoreactivity was found in the basiepithelial plexus, which is in direct contact with the flagellated cells of the epithelium (Martinez et al. 1991). Nitric oxide might modulate the activity of these cells in a way similar to that recently described for the ciliary beat frequency in airway epithelial cells (Jain et al. 1993). Furthermore, the basiepithelial plexus is near the muscle sheet of the digestive wall and NO could be involved in muscle relaxation in a similar way to the role played by NO in the motility of the mammalian gastrointestinal tract (Rand 1992). Stomach eversion is a feature unique to asteroid echinoderms. Their unusual extraoral feeding behaviour involves a process whereby the cardiac stomach is everted through the mouth and inserted between the shells of the bivalve molluscan prey (Jangoux 1982). Stomach eversion involves relaxation of the muscles of the stomach

Fig. 7A,B. Serial sections of pyloric stomach immunostained with anti-NO synthase (**A**) and with anti-NO synthase preabsorbed with the synthetic peptide (**B**) × 600. *Bar*: 20 μm

Fig. 8. Frozen section of cardiac stomach histochemically stained for NADPH-diaphorase. The precipitate is located over the basiepithelial plexus (*arrowheads*) and in vesicles of the apical region (*arrows*). × 375. *Bar*: 20 μm

wall as well as extrinsic and intrinsic retractor strands (Basch 1956). Some molecules such as ATP, adenosine and adrenaline are known to participate in this relaxation (Hoyle and Greenberg 1988; Knight et al. 1990). However, the abundance of NO synthase immunoreactivity in the pyloric stomach suggests that NO may also play a crucial role in this process.

In summary, our results clearly indicate the presence of a constitutive neuronal type enzyme in an echinoderm. Its distribution suggests that the NO generated by this enzyme has similar functions to that in mammals. Therefore, the L-arginine: NO pathway can be considered as an efficient regulatory system which developed early in the phylogenesis of the animal kingdom and has been conserved throughout evolution.

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References

Basch PF (1956) Observations on the retractor strands of the Starfish stomach. The Biol Rev City Col New York 18:14–17

Bisgrove BW, Burke RD (1986) Development of serotoninergic neurons in embryos of the sea urchin *Strongylocentrotus purpuratus*. Develop Growth and Differ 28:569–574

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 27:768–770

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 35:74–78

Cottrell GA, Pentreath VW (1970) Localization of catecholamines in the nervous system of a starfish, *Asterias rubens*, and of a brittlestar *Ophiothrix fragilis*. Comp Gen Pharmac 1:73–81

Elphick MR, Emson RH, Thorndyke MC (1989) FMRFamide-like immunoreactivity in the nervous system of the starfish *Asterias rubens*. Biol Bull 177:141–145

Elphick MR, Price DA, Lee TD, Thorndyke MC (1991) The SALMFamides: a new family of neuropeptides isolated from an echinoderm. Proc R Soc Lond [Biol] 243:121–127

Elphick MR, Riveros-Moreno V, Moncada S, O'Shea M (1993a) Identification of nitrergic neurones in invertebrates (abstract). 3rd Int Meeting on Biology of Nitric Oxide, Cologne

Elphick MR, Green IC, O'Shea M (1993b) Nitric oxide synthesis and action in an invertebrate brain. Brain Research (in press)

Forstermann U, Schmidt H, Pollock JS, Sheng H, Mitchell JA, Warner TD, Nakane M, Murad F (1991) Isoforms of nitric oxide synthase. Characterization and purification from different cell types. Biochem Pharmacol 42:1849–1857

García-Arrarás JE, Torres-Avillán I, Ortiz-Miranda S (1991) Cells in the intestinal system of holothurians (Echinodermata) express cholecystokinin-like immunoreactivity. Gen Comp Endocrinol 83:233–242

Hamid Q, Springall DR, Buttery LDK, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Riveros-Moreno V, Holgate S, Polak JM (1993) Induction of nitric oxide synthase in asthmatic patients: an immunocytochemical study of bronchial biopsies. Lancet II (in press)

Hope BT, Michael GJ, Knigge KM, Vincent SR (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88:2811–2814

Hoyle CHV, Greenberg MJ (1988) Actions of adenylyl compounds in invertebrates from several phyla: evidence for internal purinoceptors. Comp Biochem Physiol [C] 90: 113–122

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580

Huet M, Franquinet R (1981) Histofluorescence study and biochemical assay of catecholamines (dopamine and noradrenaline) during the course of arm-tip regeneration in the starfish, *Asterina gibbosa* (Echinodermata, Asteroidea). Histochemistry 72:149–154

Jain B, Rubinstein I, Robbins RA, Leise KL, Sisson JH (1993) Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. Biochem Biophys Res Commun 191:83–88

Jangoux M (1982) Digestive systems. Asteroidea. In: Jangoux M, Lawrence JM (eds) Echinoderm nutrition. Balkema, Rotterdam, pp 234–271

Knight GE, Hoyle CHV, Burnstock G (1990) Glibenclamide antagonises the responses to ATP, but not adenosine or adrenaline, in the gastric ligament of the starfish *Asterias rubens*. Comp Biochem Physiol [C] 97:363–367

Li ZS, Furness JB, Young HM, Campbell G (1992) Nitric oxide synthase immunoreactivity and NADPH diaphorase enzyme activity in neurones of the gastrointestinal tract of the toad, *Bufo marinus*. Arch Histol Cytol 55:333–350

Lyons CR, Orloff GJ, Cunningham JM (1992) Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J Biol Chem 267:6370–6374

Martínez A, López J, Villaro AC, Sesma P (1991) Choanocyte-like cells in the digestive system of the starfish *Marthasterias glacialis* (Echinodermata). J Morphol 208:215–225

Martínez A, López J, Montuenga LM, Sesma P (1993) Regulatory peptides in gut endocrine cells and nerves in the starfish *Marthasterias glacialis*. Cell Tissue Res 271:375–380

Mladenov PV, Igdoura S, Asostra S, Burke RD (1989) Purification and partial characterization of an autotomy promoting factor from the sea star *Pycnopodia helianthoides*. Biol Bull 176:169–175

Moncada S (1992) The L-arginine: nitric oxide pathway. Acta Physiol Scand 145:201–227

Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142

Nakajima Y (1988) Serotonergic nerve cells of starfish larvae. In: Burke RD, Mladenov PV, Lambert P, Parsley RL (eds) Echinoderm biology. Balkema, Rotterdam, pp 235–239

Pentreath VW, Cottrell GA (1968) Acetylcholine and cholinesterase in the radial nerve of *Asterias rubens*. Comp Biochem Physiol 27:775–785

Pollock JS, Nakane M, Buttery LDK, Martínez A, Springall DR, Polak JM, Forstermann U, Murad F (1993) Immunochemical characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. Am J Physiol (in press)

Radomski MW, Martin JF, Moncada S (1991) Synthesis of nitric oxide by the haemocytes of the american horseshoe crab (*Limulus polyphemus*). Philos Trans R Soc Lond [Biol] 334:129–133

Rand MJ (1992) Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. Clin Exp Pharmacol Physiol 19:147–169

Rimele TJ, Armstrong SJ, Grimes D, Sturm RJ (1991) Rat peritoneal neutrophils selectively relax vascular smooth muscle. J Pharmacol Exp Ther 258:963–971

Riveros-Moreno V, Beddell C, Moncada S (1993) Nitric oxide synthase: structural studies using anti-peptide antibodies. Eur J Biochem (in press)

Shirai H, Bulet P, Kondo N, Isobe M, Imai K, Goto T, Kubota I (1987) Gonad-stimulating substance of starfish. Gen Comp Endocrinol 66:50

Springall DR, Riveros-Moreno V, Buttery L, Suburo A, Bishop AE, Merrett M, Moncada S, Polak JM (1992) Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms. Histochemistry 98:259–266

Welsch U, Heinzeller T, Cobb JLS (1989) Histochemical and fine structural observations on the nervous tissue of *Antedon bifida* and *Decametra spec.* (Echinodermata: Crinoidea). Biomedical Res 10:145–154