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Nitric oxide modulates peristaltic muscle activity associated with fluid circulation in the sea pansy *Renilla koellikeri*

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Summary

Nitric oxide (NO) is a well-known regulator of vascular activities in vertebrates and it has also been implicated as a vasodilatatory agent in a cephalopod. In the sea pansy *Renilla koellikeri*, an octocorallian representative of the most basal animals with a nervous system, we investigated the role of NO in peristalsis, an activity that moves body fluids through the coelenteron (gastrovascular cavity) of the polyps across the colony. NO donors increased the amplitude of peristaltic contractions and increased tonic contractions in relaxed preparations, but caused a relaxation of basal tension in contracted preparations. The NO synthase (NOS) inhibitors L-NAME (N(ω)-nitro-*L*-arginine methyl ester) and 7-nitroindazole reduced the amplitude of peristaltic contractions and lowered basal tension. In contrast, aminoguanidine, a specific inhibitor of inducible NOS, increased the amplitude but reduced the rate of peristalsis. Zaprinast, a cGMP-specific phosphodiesterase inhibitor, decreased the amplitude of peristaltic contractions, a decrease that was amplified by dibutyryl cGMP. In contrast, the inhibitor of soluble guanylyl cyclase ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) enhanced peristalsis. Putative NOS-containing neurons, revealed by NADPH-diaphorase activity and citrulline immunohistochemistry, were observed in the basiectoderm at the base of the autozooid polyp tentacles and in a nerve-net around the oral disc. Their neurites ran up the tentacles and down to the polyp body wall, crossing from the ectoderm through the mesoglea and into the endoderm musculature where musculo-epithelial cells were also reactive. These data suggest that two distinct nitrergic pathways, one of which is mediated by cGMP, regulate peristalsis and muscle tone in the sea pansy and that these pathways may involve NOS-containing ectodermal neurons and musculo-epithelial cells.

Key words: sea pansy, Cnidaria, peristalsis, muscle, nitric oxide (NO).

Introduction

Nitric oxide (NO) has emerged in the last two decades as an important signalling molecule with various functions in both vertebrates and invertebrates (Jacklet, 1997; Colasanti and Venturini, 1998; for a review see Moroz, 2001). In many invertebrates, and particularly in mollusks (Moroz et al., 1993; Elphick et al., 1995; Moroz, 2000; Gelperin et al., 2000; Cole et al., 2002) and in arthropods (Truman et al., 1996; Muller, 1997; Scholz et al., 1998, 2001), NO synthase (NOS) activity is concentrated in the nervous system except that of the salivary gland of a blood-feeding insect (Ribeiro and Nussenzveig, 1993) and the firefly lantern (Trimmer et al., 2001). As a neuroactive agent, NO has been widely implicated in neuronal development, chemosensory processing and the control of feeding (Moroz, 2001).

That NO is a phylogenetically ancient signalling molecule in multicellular animals is documented by reports of the presence and activity of NO systems in cnidarians, the most basal animals with nervous systems. NO has been implicated in the regulation of cnidocyte discharge in the sea anemone *Aiptasia diaphana* (Salleo et al., 1996), in the feeding response of *Hydra vulgaris* to chemosensory stimuli (Colasanti et al., 1997), and in the response to stress of *Aiptasia pallida* (Trapido-Rosenthal et al., 2001). While Elofsson et al. (1993) found no evidence of NOS activity in cnidarian neurons, NOS activity was detected by NADPH diaphorase staining in unidentified cells of the ectoderm and endoderm of the body wall of *A. diaphana* (Morrall et al., 2000). Only recently has NOS activity been demonstrated in sensory neurons of the jellyfish *Aglantha digitale* in which NO was reported to activate motoneurons and to upregulate swimming (Moroz et al., 2004).

While regulation of vascular tone was the first role for NO to be reported in vertebrates (for a review, see Cosentino and Lüscher, 1996), such a role was rarely identified in invertebrates. A vasodilatatory role was reported in the cephalopod *Sepia officinalis* (Schipp and Gebauer, 1999), and the only effect of NO that may present some analogy to vascular control in another invertebrate is the relaxation of

smooth muscles in the starfish *Asterias rubens* (Elphick and Melarange, 1998; Melarange and Elphick, 2003). Although cnidarians are basically bilayered (diploblastic) animals without separate circulatory and gastric organs, the sea pansy *Renilla koellikeri*, an octocorallian of the sea pen family, generates peristaltic contractions that are the driving force for the movement of sea water through the gastrovascular cavity (coelenteron) of polyps and into the colonial mass (Parker, 1920). Sea water is pumped in through the pharynx of numerous inhalent siphonozooids, circulates through gastrovascular cavities, reaches the axial canal and is pushed out through the large exhalent siphonozooid (Fig. 1). The gastrovascular cavities of the colony are lined by musculo-epithelial cells in which smooth muscle fibres are laid down largely in circular or longitudinal orientations, and by gastric cells, which secrete enzymes that digest food particles flowing by (Lyke, 1965). Thus the internal channels of the sea pansy combine gastric and circulatory roles, thereby making this anthozoan an attractive model to investigate the physiology of a primitive gastrovascular system.

Regulatory activities of peristalsis in the sea pansy have previously been investigated. Serotonin was reported to enhance peristaltic activity through the mediation of cyclic AMP (Anctil, 1989). In contrast, melatonin sharply depressed peristalsis through the mediation of cyclic GMP (Anctil et al., 1991). Peptides of the gonadotropin-releasing hormone family also depressed peristalsis (Anctil, 2000). Neurons were labelled by antibodies raised against these putative transmitters (Umbriaco et al., 1990; Mechawar and Anctil, 1997; Anctil, 2000). In this study, we tested whether NO is a regulator of peristalsis and muscle tone in the sea pansy by analogy to the role of NO in modulating vascular tone in vertebrates and in cephalopods. To achieve this, first, we recorded the effects of NO donors and NO-related drugs on peristaltic contractions of reduced sea pansy preparations and on the contractile state of intact animals. Second, we examined the distribution of putative NOS activity in the tissues of the sea pansy by NADPH-diaphorase histochemistry and citrulline immunohistochemistry. Preliminary results from this work were presented at the sixth International Congress of Comparative Physiology and Biochemistry (Anctil and Poulain, 2003).

Materials and methods

Animals

Colonies of the sea pansy *Renilla koellikeri* (Pfeffer) were collected by divers at various sites along Southern California shores and shipped to Montreal initially by Marinus Inc. (Long Beach, CA, USA), and since September 2003 by Marinus Scientific (Garden Grove, CA, USA). The animals were held in filtered, artificial sea water (ASW; Instant Ocean Aquarium Systems, Mentor, OH, USA) on a 12 h:12 h light:dark cycle. Aquarium temperature ranged between 14 and 21°C depending on season. Animals were not fed and were used after a minimum of 24 h following arrival of animal shipments.

Physiological and behavioural experiments

Peristaltic contractions were recorded from reduced preparations as described by Anctil (1989) with modifications. Polyp bearing triangular pieces of the colony mass were excised consistently from the same area in each of the colonies used. Two cuts through the entire thickness of the colonial mass were initiated from the median axial canal proceeding to the left outer margin of the colony, thus resulting in a triangular piece with the outer margin intact (Fig. 1A). The outer margin of the preparation was pinned on Sylgard coating (Dow Corning Canada, Mississauga, ON, Canada) near the bottom of a 50-ml experimental bath, and the wedge tip, opposite the outer margin (originating from the axial canal), was attached with thread to a Grass FT-03C isometric force transducer (Astro-Med Inc, Longueuil, Canada). The transducer signals were transmitted to a Grass CP122 strain gauge amplifier that was interfaced with a Grass PolyView analogue-to-digital converter and data acquisition system. The calibrated mass values were converted to force units (Newtons). In a few experiments designed to monitor the excitability of preparations, two 30G Grass platinum electrodes were inserted into reduced preparations and were fed to a Grass S48 square pulse stimulator (Astro-Med Inc.).

Because manipulations led to extremely contracted preparations, these were allowed to relax in the bath with fresh ASW for 30–90 min before experiments. Bath solutions were routinely maintained at 21–23°C. As the basal tension dropped, peristaltic waves began to appear and they reached relatively stable amplitudes when basal tension itself stabilised. Typically, experiments began by recording peristaltic activity for 30 min after adding a volume of solvent (filtered ASW alone or ASW with 1% dimethylsulfoxide) equal to that used later when adding drug solutions. Drugs were added and peristaltic activity was recorded for another 30 min. This was usually followed by a rapid and complete evacuation of the bath, which was then filled with fresh ASW. When the amplitude of peristaltic waves stabilized after washing, the same drug was added again at the same or a different concentration, or a putative antagonist drug was added, and peristaltic activity recorded for 30 min. When an antagonist drug was used, addition of the first drug followed the incubation with the antagonist, without washing for 30 min, then the bath contents were replaced with fresh ASW a second time. A period of 1 h after washing was allowed before the first drug was added a last time, and peristaltic activity was monitored to assess reversibility of drug effects. Amino-3-morpholinyl-1,2,3-oxadiazolium (SIN-1) chloride, aminoguanidine hydrochloride, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) and Zaprinast were obtained from Tocris Cookson Inc. (Ellisville, Missouri, USA). All remaining drugs mentioned below were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada) unless stated otherwise.

Although SIN-1 is known to generate superoxide ions as well as NO (Feelisch et al., 1989; Hogg et al., 1992), we used substantially lower concentrations of SIN-1 than those used to

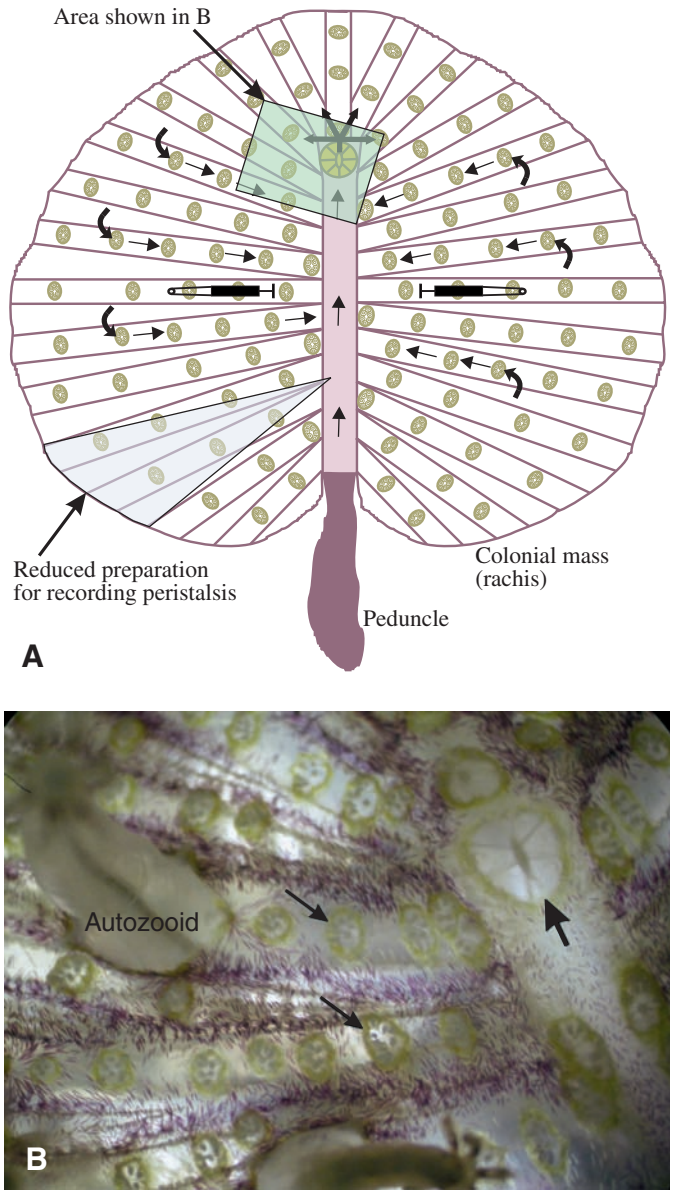

Fig. 1. Morphological organization of sea pansy colonies with emphasis on water circulation. (A) Schematic diagram of the upper surface of the discoid colony showing the distribution of water polyps (siphonozooids, shown in green) and the direction of water flow in the channels beneath the polyps (arrows). Water from the channels and the peduncle reaches the axial canal and moves forward to the large exhalent siphonozooid (boxed region, shown in B) where it is ejected. The triangle shaded in blue represents the area excised for reduced preparation recording. Syringe symbols represent site and orientation of drug (left) and vehicle (right) injections for behavioural experiments. The autozooids (feeding and reproductive polyps) are not represented for better clarity. (B) Photomicrograph of area corresponding to the boxed region in A, from a living colony. Note the autozooid, the miniature inhalent siphonozooids (small arrows) and the single exhalent siphonozooid (large arrow) in which the eight septa irradiating from the mouth opening are typical of the octocorallian body organization. Note also the masses of spicules (shown in violet) forming the calcified skeleton of the colony.

detect superoxides (1-5 mmol l^{-1}). In addition, the effects of SIN-1 on our preparations were similar to those induced by S-nitroso-*N*-acetylpenicillamine (SNAP), a NO donor that does not generate superoxide ions (see Results).

Monitoring experimental conditions for 30 min each allowed the passage of five to eight peristaltic waves. From the data analysed with the Grass Polyview software, the amplitudes of the last three peristaltic contractions for each condition were averaged. The averaged value for each preparation exposed to the solvent (vehicle) was subtracted from that of the same preparation during drug exposure, and the differential was normalized as a percentage relative to the control value (vehicle). Hence data are represented as means ± standard error of change in peristaltic force relative to controls, where N is the number of separate experiments on different reduced preparations. Statistical significance between any two sets of data was evaluated by using the paired t-test or the Mann–Whitney test.

In addition to reduced preparations, whole colonies were used to record the effects of NO-related drugs on contractile behaviour. The colonies were placed individually in 140×75 mm crystallizing dishes filled with ASW maintained at 21–23°C and allowed to relax. Drugs were injected gently, under a dissecting microscope, with a syringe and a 26G needle into a gastrovascular channel on the left side of the biradial colony as shown in Fig. 1A. An identical volume of solvent was similarly injected in an opposite channel on the right side of the colony. Thus each colony served as its own control. Successful experiments resulted in long-lasting (>1 h) shape asymmetries of the two sides (see Results). Preliminary injections of Methylene Blue had shown that periods in excess of 40 min were needed for dye to visibly transfer from one side of the colony to the other.

NADPH-diaphorase histochemistry

Although immunohistochemistry with Universal NOS (uNOS) antibodies was successful in higher invertebrates (Scholz et al., 2002; Christie et al., 2003), its use (product no. PA1-039; Affinity BioReagents, Golden, CO, USA) in the sea pansy at a dilution of 1:100 and incubation time of 48 h gave no result. This may be due to a lack of significant homology between vertebrate and cnidarian NOS isoforms. Therefore, fixative-resistant NADPH–diaphorase (NADPH–d) activity was used instead, as a marker for NOS activity.

The NADPH–d histochemical method of Moroz et al. (2004) was applied as follows. The distal segment (anthocodium) of autozooid (feeding) polyps was excised from colonies, some of which were anaesthesized in a 1:1 mixture of 0.37 mol l^{-1} $MgCl_2$ and ASW and the anaesthetics rinsed off before further processing. In addition, mesenteries were dissected and removed from inside the colonial mass (rachis). The tissues were fixed in 4% paraformaldehyde in ASW (pH 7.8) for 30 min at room temperature. They were rinsed 3×10 min in 0.5 mol l^{-1} Tris-HCl (pH 8). Staining took place in a solution containing 1 mmol l^{-1} β-NADPH, 0.5 mmol l^{-1} nitro blue tetrazolium, 0.3% Triton X-100 and 0.5 mol l^{-1} Tris-HCl. The

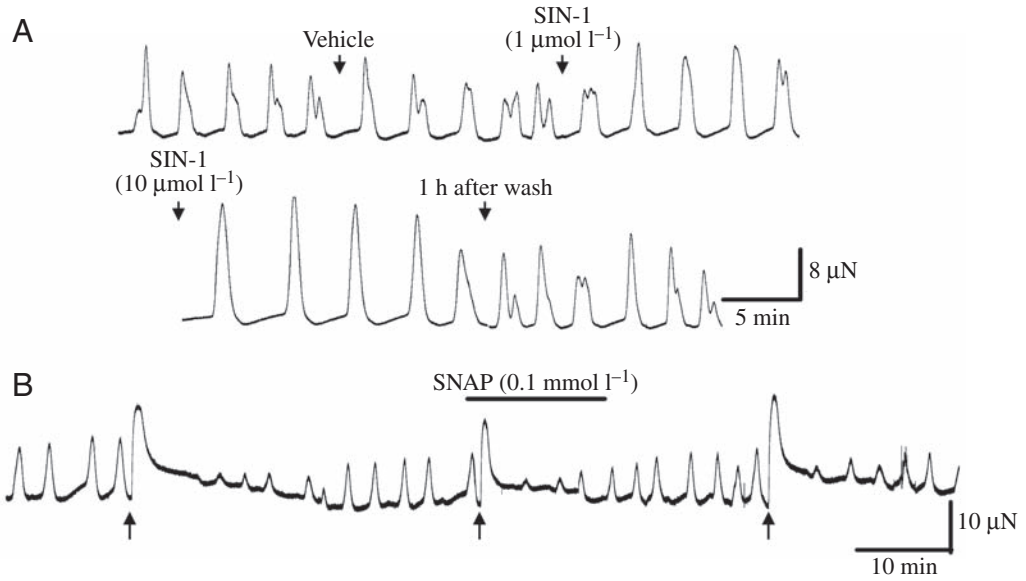

Fig. 2. Recordings of effects of NO (nitric oxide) donors on peristaltic contractions of reduced preparations of *R. koellikeri*. (A) Recording of a single preparation immersed in 40 ml of ASW and first exposed to 40 ml of solvent (ASW), then consecutively to 40 and 400 µl of 1 mmol l^{-1} SIN-1 (amino-3-morpholinyl-1,2,3-oxadiazolium) chloride to obtain final bath concentrations of 1 and 10 µmol l^{-1}, respectively. Note the diversity of shape of traces reflecting the contribution of different contraction waves to peristaltic events. Note also the dose-dependent increase in contraction amplitudes and in wave synchrony after exposure to SIN-1 and the reversibility of this response after washing. (B) Effect of SNAP (S-nitroso-*N*-acetylpenicillamine) on contractions induced by field electrical stimulation. Stimulation trains of 6 monopolar pulses of 10 ms each at 5 V and 2 Hz were applied as shown at arrows. Note that the induced robust contractions were invariably followed by a sharp, transient drop of the ongoing peristaltic waves. Note also the reversible drop in amplitude and duration of the induced contraction in the presence of SNAP. µN, microNewton.

incubation lasted 90 min in the dark at room temperature, followed by three rinses of 10 min each in 0.5 mol l^{-1} Tris-HCl. Tissues were post-fixed in 4% paraformaldehyde in 50% methanol for 30–60 min, followed by dehydration in two changes of ethanol of 15 min each. Tissues were cleared in xylene and mounted in Permount (Fisher Scientific).

Controls were performed by substituting β-NADH or β-NADP+ for β-NADPH at the same concentration. Additionally, diphenyleneiodonium, a selective inhibitor of specific NADPH-dependent activity, was used at a concentration of 0.1 mmol l^{-1}.

Citrulline immunohistochemistry

As another alternative approach to the localization of NOS, an anti-citrulline antibody was used. Citrulline is a by-product of NO formation from arginine and therefore citrulline production is considered a reliable indicator of NOS enzymatic activity (Eliasson et al., 1997; Scholz et al., 2001).

For sectioned preparations, the colonial mass was rapidly cut into slices ≈5 mm thick that were oriented parallel to the biradial axis of the rachis (see Fig. 1A). The slices were immersed for 2 h at 4°C in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (PB). The slices were washed 3× 15 min in phosphate buffered saline (PBS; 2.4% NaCl added to PB), then transferred to 15% and 30% sucrose in PBS for 30 min each. The slices were placed in Tissue-Tek OCT, frozen in dry ice-chilled isopentane and sectioned at 16 mm with a Leica CM 3050 cryotome (Leica Microsystems, Dollard-des-Ormeaux, Quebec, Canada). Sections were placed on gelatin–chromalum-coated slides and frozen at –20°C until processed.

Sections were washed 3× 15 min in PBS containing 0.2% Triton X-100 (PBST), followed by 10 min in PBS containing 1% H_2O_2 to inactivate endogenous peroxidases. After another rinse in PBST the sections were transferred to a diluted (1:1000 in PBST) solution of a polyclonal antibody raised in rabbit against a citrulline–glutaraldehyde–BSA complex (AB5612, Chemicon International, Temecula, CA, USA). Sections were incubated overnight in the primary antibody at 4°C. They were washed again repeatedly and processed with the BioStain Super ABC/peroxidase rabbit IgG detection kit according to the manufacturer's instructions (Biomeda, Foster City, CA, USA). After repeated washes in PBS, section staining was completed with 0.06% 3,3′-diaminobenzidine in PBS intensified with 0.025% cobalt chloride and 0.02% nickel ammonium sulphate. Sections were immersed first in DAB alone for 10 min, followed by DAB with 0.01% H_2O_2 for another 10 min. After a 15 min wash in PBS the sections were mounted in a glycerol–PBS mixture and stored at –20°C until examined.

For whole mounts, tissues were excised from colonies as described in the preceding section. Tissue processing followed the procedure for sections except that PBST contained 0.4% Triton X-100 and that the duration of washes was doubled. In all experiments preparations in which the primary antibody step was omitted served as controls. In addition, some preparations were treated with the NOS inhibitor 7-nitroindazole, at 0.1 mmol l^{-1} for 3–6 h before fixation and citrulline immunohistochemistry. The latter control revealed non-specific diffuse staining throughout the tissues and in some cells of the mesenteries, but otherwise the staining abolished by 7-nitroindazole was consistent with specific NADPH-d staining (see Results).

Microscopy and image treatment

Whole-mounts and sections were viewed with a Wild-Leitz Laborlux S trinocular microscope and photographed with a Nikon Coolpix 4500 digital camera. Images were cropped and contrasted with Adobe Photoshop. Colour enhancement was also applied for some images (Figs 8A-D, 9A,C).

Results

Effects of NO donors

The NO donor SIN-1 increased the amplitude of peristaltic contractions in reduced preparations (Fig. 2A). The response appeared within 2–5 min after addition of SIN-1. Furthermore, SIN-1 largely synchronized the different phases of peristaltic waves, such that the recorded double-peak contractions in control conditions became single-peak in the presence of SIN-1 (Fig. 2A). The response was dose-dependent and peaked at 50 mmol l^{-1}, and it was largely reversible after washout (Fig. 2A, Fig. 3A). The NO donor SNAP had effects similar to SIN-1 on peristalsis, and the SNAP-induced increase of peristaltic force was significantly reduced in the presence of the serotonin blocker 1-(1-naphthyl)piperazine HCl (Fig. 3A). The NO donors had no consistent effect on the frequency and duration of peristaltic contractions.

Injection of NO donors into the gastrovascular cavities of the colonial mass of five intact colonies in a relaxed state consistently induced a contracted state that occurred after 3–6 min and lasted for at least 1 h (Fig. 3B). During that period, the response was confined to the half of the biradial colony where the injection occurred. The other half, injected with the vehicle alone, remained in a relaxed state (Fig. 3B). Both halves of the colony displayed peristalsis during this procedure. In contrast, injecting four colonies in a fully contracted state with NO donors consistently caused a relaxation of the half colony where injection occurred, while the half injected with the vehicle remained contracted (Fig. 3C). In addition, the contractile responses of reduced preparations to electrical stimulation were decreased by the NO donor SNAP (Fig. 2B). The amplitude, duration and relaxation phase of these responses were diminished by the NO donor in a reversible manner. Because peristaltic activity was undetectable in fully contracted colonies and recordings of reduced preparations show a sharp drop of peristaltic contraction amplitude after electrically induced contractions (Fig. 2B), relaxing effects of NO donors on peristalsis were not investigated.

Effects of NOS inhibitors

The competitive NOS inhibitor L-NAME (N(ω)-nitro-L-arginine methyl ester) decreased the amplitude of peristaltic contractions and substantially lowered basal tension in a dose-dependent manner (Fig. 4, Fig. 5A). The effect of 0.1 mmol l^{-1} L-NAME on peristalsis was entirely reversible over a period of 1 h following washout, but basal tension did not return to control levels during that period. The stereospecific isomer D-NAME had no significant effect on these activities (Fig. 5A). Another NOS inhibitor, 7-nitroindazole, had an effect similar to that of L-NAME (not shown). In contrast, aminoguanidine, a selective inhibitor of inducible NOS (iNOS) (Griffiths et al.,

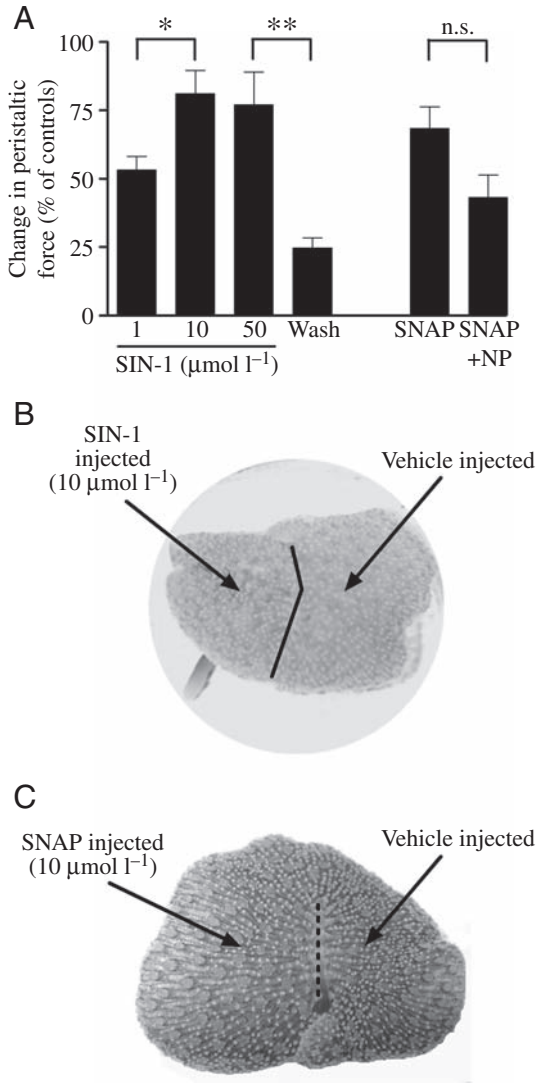

Fig. 3. Quantitative summary of effects of NO donors on peristalsis (A) and representative effects on NO donors on the behaviour of whole colonies (B,C). (A) Histogram showing the change, relative to controls, in the amplitude of peristaltic contractions induced by the NO donors SIN-1 (amino-3-morpholinyl-1,2,3-oxadiazolium) and SNAP (S-nitroso-*N*-acetylpenicillamine). Note that the change is largely reversible after exposure of reduced preparations to 50 μmol l^{-1} SIN-1. Statistical significance: **P*<0.05 or ***P*<0.01 with the Mann–Whitney test. Note that 10 μmol l^{-1} 1-(1-naphthyl)piperazine (NP) reduced the increase in peristaltic force induced by 10 μmol l^{-1} SNAP, but the difference was not significant (n.s., *P*>0.05). *N*=10 (1 μmol l^{-1} SIN-1), 6 (10 μmol l^{-1}), 4 (50 μmol l^{-1}) and 8 (wash), 6 (SNAP) and 4 (SNAP + NP). (B) Injection of SIN-1 in the left half of an initially relaxed colony led to a contracted state relative to the control (right) half. Black line indicates the position of the axial canal. (C) Injection of SNAP in the left half of an initially contracted colony led to a relaxed state relative to the control half.

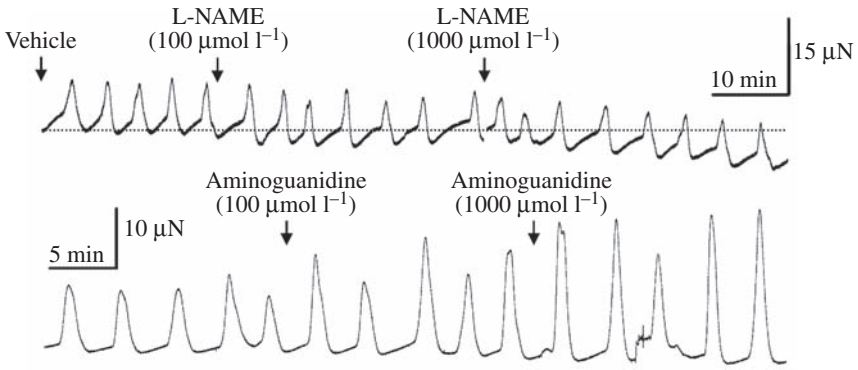

Fig. 4. Recordings of effects of NOS inhibitors on peristaltic contractions of reduced preparations. L-NAME (N(ω)-nitro-*L*-arginine methyl ester) dose-dependently reduced both the amplitude of peristaltic waves and basal tension whereas aminoguanidine, in a separate preparation, increased peristaltic waves without affecting basal tension. Dotted line indicates initial basal tension.

1993; Joly et al., 1994), increased considerably the amplitude of peristaltic contractions without affecting basal tension (Fig. 4). This effect was dose-dependent (Figs 4, 5A) and reversible 35–45 min after washout.

Injection of 1 mmol l^{-1} L-NAME induced a relaxed state in four of six contracted intact colonies tested, whereas the site of injection of the solvent vehicle remained contracted (Fig. 5B). The effect appeared 5–8 min after injection and began to wane 50–70 min after injection. In contrast, injection of 1 mmol l^{-1} aminoguanidine led to a contracted state within 6–10 min after injection in all six relaxed colonies tested (Fig. 5C). This effect vanished 30–40 min after washout. While peristalsis was observed in all the colonies tested with either NOS inhibitor, it was more robust in aminoguanidine-treated colonies.

To test whether the potentiating effect of serotonin on the amplitude of peristaltic contractions of reduced preparations (Anctil, 1989) could be mediated by NO, the NOS inhibitor L-NAME was added to the bath and the response to serotonin (5-hydroxytryptamine creatinine sulphate) recorded 30 min later. Serotonin at 10 mmol l^{-1} caused a 75% increase in the amplitude of the contractions, and this increase was reduced to near 20% in the presence of 0.1 mmol l^{-1} L-NAME (Fig. 5A). Similarly, the ability of aminoguanidine to interfere with the inhibitory effect of melatonin on peristaltic contractions (Anctil et al., 1991) was tested. Melatonin, at 10 mmol l^{-1}, reduced the amplitude of the contractions by more than 60%, and this reduction was not significantly changed in the presence of 1 mmol l^{-1} aminoguanidine (Fig. 5A). In contrast, aminoguanidine not only eliminated the inhibiting effect of mammalian GnRH (gonadotropin-releasing hormone), which, similarly to GnRH-like sea pansy factors, reduces peristalsis (Anctil, 2000), but also reversed the response to produce a potentiating effect on peristalsis (Fig. 5A).

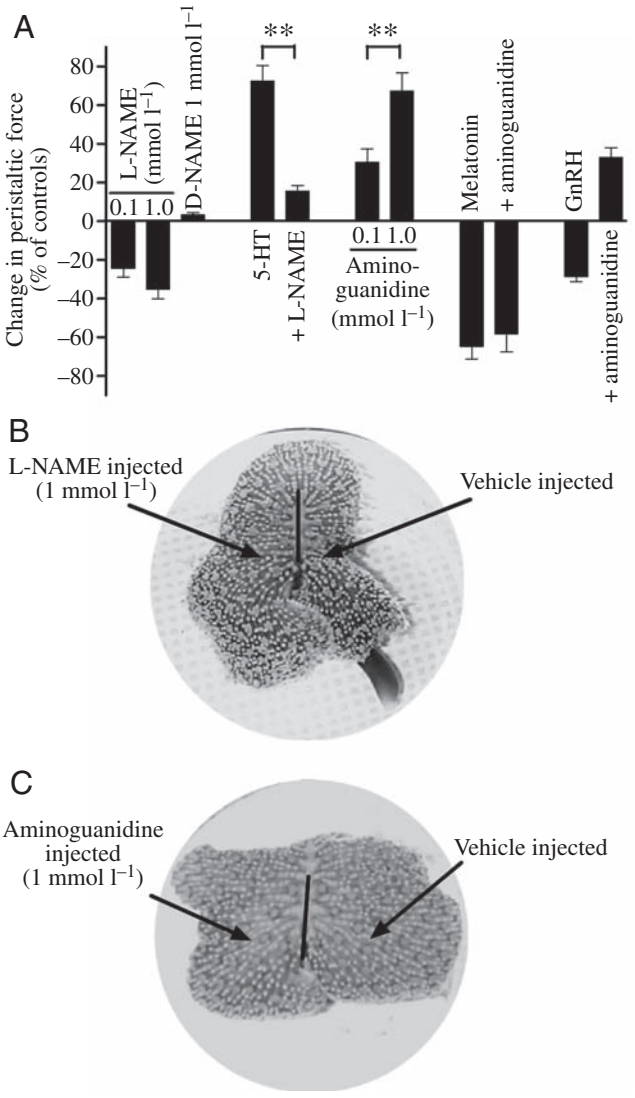

Fig. 5. Quantitative summary of effects of NOS inhibitors on peristalsis (A) and representative effects of NOS inhibitors on the behaviour of whole colonies (B,C). (A) Histogram showing the actions of NOS inhibitors on peristalsis as well as the interactions between NOS inhibitors and conventional transmitter effects (see Results). Statistical significance: $^{**}P<0.01$with a paired *t*-test. There was no significant difference between 0.1 and 1.0 mmol l^{-1} L-NAME (N(ω)-nitro-*L*-arginine methyl ester) or between melatonin alone and with aminoguanidine. *N*=6 (L-NAME), 3 (D-NAME), 5 (aminoguanidine), 4 (melatonin/aminoguanidine and GnRH/aminoguanidine). (B) Injection of L-NAME in the left half of a contracted colony led to a relaxed state relative to the control (right) half. (C) Injection of aminoguanidine in the left half of a relaxed colony led to a contracted state relative to the control half. Black line indicates the position of the axial canal.

Effects of inhibitors of soluble guanylyl cyclase and cGMP-specific phosphodiesterase

ODQ (at 0.1 mmol l^{-1}), a specific blocker of NO-dependent guanylyl cyclase, rapidly increased the amplitude of peristaltic contractions by 65% in reduced preparations (Fig. 6, Fig. 7A).

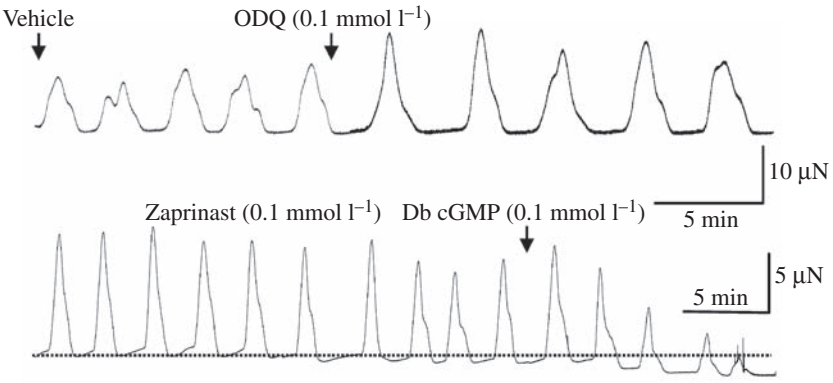

Fig. 6. Recordings of effects of the soluble guanylate cyclase inhibitor ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) and of cGMP-specific phosphodiesterase inhibitor zaprinast on peristaltic contractions of reduced preparations**.** Note the enhancing effect of ODQ in contrast to the additive depressing effects of zaprinast and db cGMP. The latter two also depressed basal tension below initial level (dotted line).

In the presence of 0.1 mmol l^{-1} SNAP, ODQ at the same concentration increased this amplitude by a further 20%, although the difference is not significant (P>0.01, paired t-test). The inhibiting effect of melatonin, via cyclic guanosine monophosphate (cGMP), on peristalsis was previously reported (Anctil et al., 1991). In the present study, melatonin at 10 mmol l^{-1} reduced the amplitude of contractions by 60%, and this reduction was halved in the presence of 0.1 mmol l-1 ODQ (Fig. 7A). Zaprinast, a selective inhibitor of cGMP-specific phosphodiesterase, reduced the amplitude of peristaltic contractions and the addition of dibutyryl cGMP (db cGMP) further reduced this amplitude (Fig. 6, Fig. 7A). This response was accompanied by a slight drop in the basal tension. The response to both zaprinast and db cGMP appeared 6–7 min after exposure and was fully reversible after washout. Similarly, IBMX, which appears to act as a selective cGMP phosphodiesterase inhibitor in the sea pansy (Anctil et al., 1991), sharply reduced the amplitude of peristaltic waves beyond the level of reduction achieved with zaprinast (Fig. 7A).

Injection of 0.1 mmol l^{-1} ODQ into six intact colonies consistently induced a contracted state and strong peristalsis compared to the control side of the colonies (Fig. 7B). In contrast, 0.1 mmol l^{-1} zaprinast, injected in the left half of five intact colonies, consistently relaxed that half and reduced its peristalsis, whereas the control right half was more contracted and underwent strong peristalsis (Fig. 7C).

Distribution of putative NOS-containing cells

The sea pansy is a colony of polyps: the autozooids are the feeding and reproductive polyps, and the siphonozooids control the intake and exit of water (Fig. 1). The part of the authozooids that emerges above the colonial mass, the anthocodium, includes the upper body column, the tentacles and mouth (oral disc). Histochemical staining of NADPH-d was localized throughout the anthocodium. In addition, staining was observed in endodermal muscles of the autozooid parts buried inside the colonial mass (zooecium) and in the perinuclear region of oocytes. No staining was detected in preparations in which β-NADPH was replaced by β-NADH or β-NADP+, and staining was abolished in preparations exposed to diphenyleneiodonium except for oocytes where staining remained.

Staining was prominent in the oral disc (Fig. 8A) and at the base of tentacles of autozooids (Fig. 8B). Staining was also present in small cells (4–6 μm) aggregated at the edge of the oral disc and a few other cells scattered over the surface of the oral disc where there is also a dense meshwork of fine neurites (Fig. 8C). Although processes emerge from these cells, the relationship of the latter to the neurite meshwork could not be ascertained. In tentacles, many stained neurites run alongside each other from the tentacle base to the tentacle tip both on the oral and aboral sides. The fine processes are varicose and intertwine with each other (Fig. 8D). The neurites on the aboral side of tentacles extend into the upper body column where they merge with neurites from the neighbouring tentacle to form a local nerve-net (Fig. 8B,D). The somata of the tentacle cells are localized at the base of the ectodermal epithelium (Fig. 8E,F) where a basiectodermal nerve-net is located (Lyke, 1965; Fautin and Mariscal, 1991). Neurites from the oral side of tentacles extend into the oral disc (Fig. 8F). The stained tentacle cell somata are small (4–6 μm) and bear an apical process typical of cnidarian neurosensory cells (Fig. 8G). Because of their basiectodermal position, they possess a short process that bifurcates within the neurite meshwork near the ectoderm-mesoglea interface (Fig. 8F) where an ectodermal muscle layer is located (Lyke, 1965; Fautin and Mariscal, 1991). The cell somata in the oral disc have a similar morphology except that the presence of an apical process could not be clearly ascertained.

The distribution of 7-nitroindazole-sensitive citrulline immunostaining was similar to that of NADPH-d staining. In particular, immunostained cells were present in the same location at the base of tentacles (Fig. 9A) as for the NADPH-d reactive cells (Fig. 8E). In cross-sections of the tentacles, the immunostaining was concentrated at the base of the ectoderm and in the endoderm just across from the mesoglea (Fig. 9B).

Fig. 8E shows a bundle of NADPH-d stained neurites running from cell somata at the base of a tentacle downward to the body column. In autozooid polyps the body column possesses eight radial septa that compartmentalize eight gastrovascular cavities between the outer wall and the central pharynx. These cavities line up with the cavity of each of the eight tentacles above them. The stained neurite bundles were localized at the interface of each septum with the outer wall (Fig. 9C,D). All these cavities are covered by endodermal epithelia in which musculo-epithelial cells are present. In the outer wall endoderm these cells form circular muscle sheets that consistently display NADPH-d staining (Fig. 9D).

Citrulline immunostaining was also found in this circular musculature, both in the muscle feet of the myoepithelial cells themselves and in multipolar neurons that send processes to these feet (Fig. 9E). These endodermal neurons do not appear to be interconnected. Occasionally NADPH-d staining was also present in the muscular septa where muscle fibre orientation differs from that of the circular musculature (Fig. 9D). A similar localization of citrulline immunostaining was observed. In sectioned material of zooecia, which contribute the bulk of endodermal muscles involved in transcolonial peristaltic activity, citrulline immunostaining was detected in both septal muscles (longitudinal and radial), as well as in the circular muscle of the outer wall (Fig. 9F). However, it was not possible to ascertain whether staining was present inside the musculo-epithelial cells because of the interfering presence of neurites over the muscle elements.

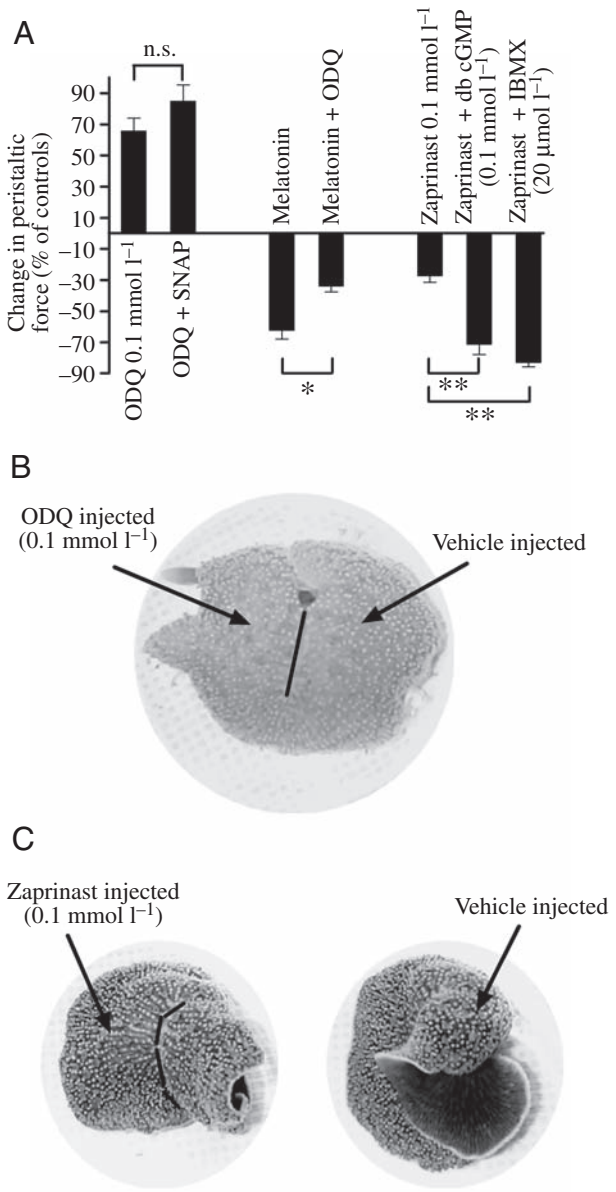

Fig. 7. Quantitative summary of effects of guanylate cyclase and phosphodiesterase inhibitors [ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) and zaprinast, respectively] on peristalsis (A) and their representative effects on the behaviour of whole colonies (B,C). (A) Histogram showing the actions of ODQ and zaprinast on peristalsis, as well as their interactions with the effects of other signalling molecules (see Results). Statistical significance: $P>0.05$ (n.s.), *$P<0.05$ or **$P<0.01$with the paired *t*-test. $N=6$ (ODQ/SNAP), 4 (melatonin/ODQ) and 5 (zaprinast/db cGMP/IBMX). (B) Injection of ODQ in the left half of the colony led to a slightly contracted state and stronger peristalsis (as illustrated by the marginal fold) relative to the control (right) half. (C) Injection of aminoguanidine in the left half of another colony led to a relaxed and quiescent state relative to the control half where robust peristalsis occurs (compare the two pictures of the same colony photographed 30 s apart). Black line indicates the position of the axial canal.

Discussion

Nitrergic signalling in the sea pansy

The data presented here suggest that NO may act as a modulator of peristaltic activity and of basal muscle tension in the sea pansy. This is supported by the dose-dependent, potentiating effect of the NO donors SIN-1 and SNAP on the amplitude of peristaltic contractions of reduced preparations and on changes of basal tension level induced by these agents in intact colonies. Peristaltic waves appear as variable multi-phase events that reflect the contribution of different muscles generating them. The shifting of peristaltic waves from multi-phase to single-phase in the presence of NO donors (Fig. 2A) points to a synchronization of the contributing muscles that may be due to a direct role of NO in phase setting of the peristalsis pacemaker or, more likely, to the paracrine diffusion of NO through the muscular epithelia.

In addition, the pharmacological data with NOS inhibitors suggest that NO is endogenously generated and that two distinct nitrergic pathways may be involved. The reduction of the amplitude of peristaltic contractions and of basal tension levels by L-NAME and 7-nitroindazole is consistent with the stimulatory effects of NO donors on these activities. In contrast, the potentiating effect of a selective inhibitor of iNOS, aminoguanidine, on the amplitude of peristaltic contractions appears to be inconsistent with these effects. Interestingly, the induction of a contracted state in intact colonies by aminoguanidine is the opposite of the relaxing effect of NO donors on the initially contracted colony. This suggests that two NOS isoforms exist in the sea pansy, one of which may be a NOS involved in a pathway leading to a positive modulation of peristalsis and basal tension, and the other could be a iNOS associated with general muscle relaxation and negative modulation of peristalsis. Although this hypothesis must be validated by the characterization of NOS forms in the sea pansy, the recent cloning of an iNOS isoform in another anthozoan, the corallimorph *Discosoma* (Panchin, Sadreyev and Moroz, personal communication in Moroz et al., 2004), makes it plausible.

The possibility that iNOS-like activity in the sea pansy is involved in regulating peristalsis raises interesting

physiological issues. This form of NOS commonly shows negligible activity unless induced by agents such as cytokines and bacterial endotoxins that set in motion defence mechanisms against pathogens (Griffin and Stuehr, 1995; Moroz, 2001). The consistent effect of aminoguanidine on peristalsis suggests a similar role of iNOS in the sea pansy, and points to the existence of dual NOS synthetic pathways (constitutive *versus* inducible) as in vertebrates. However, only constitutive, neuronal forms of NOS have been reported so far in other invertebrates (Moroz, 2001).

Interactions of NO with other transmitters

Because the indoleamines serotonin and melatonin were reported to modulate the peristalsis of the sea pansy in opposite manners (Anctil, 1989; Anctil et al., 1991), the possibility arose that the effects of NO on peristalsis are either mediated by the indoleamines or constitute the mediation event for the responses to indoleamines. The sharp diminution of the potentiating effect of serotonin on peristalsis by L-NAME (Fig. 5A) suggests that NO mediates the serotonin response. Conversely, the serotonin antagonist 1-(1-naphthyl)piperazine,

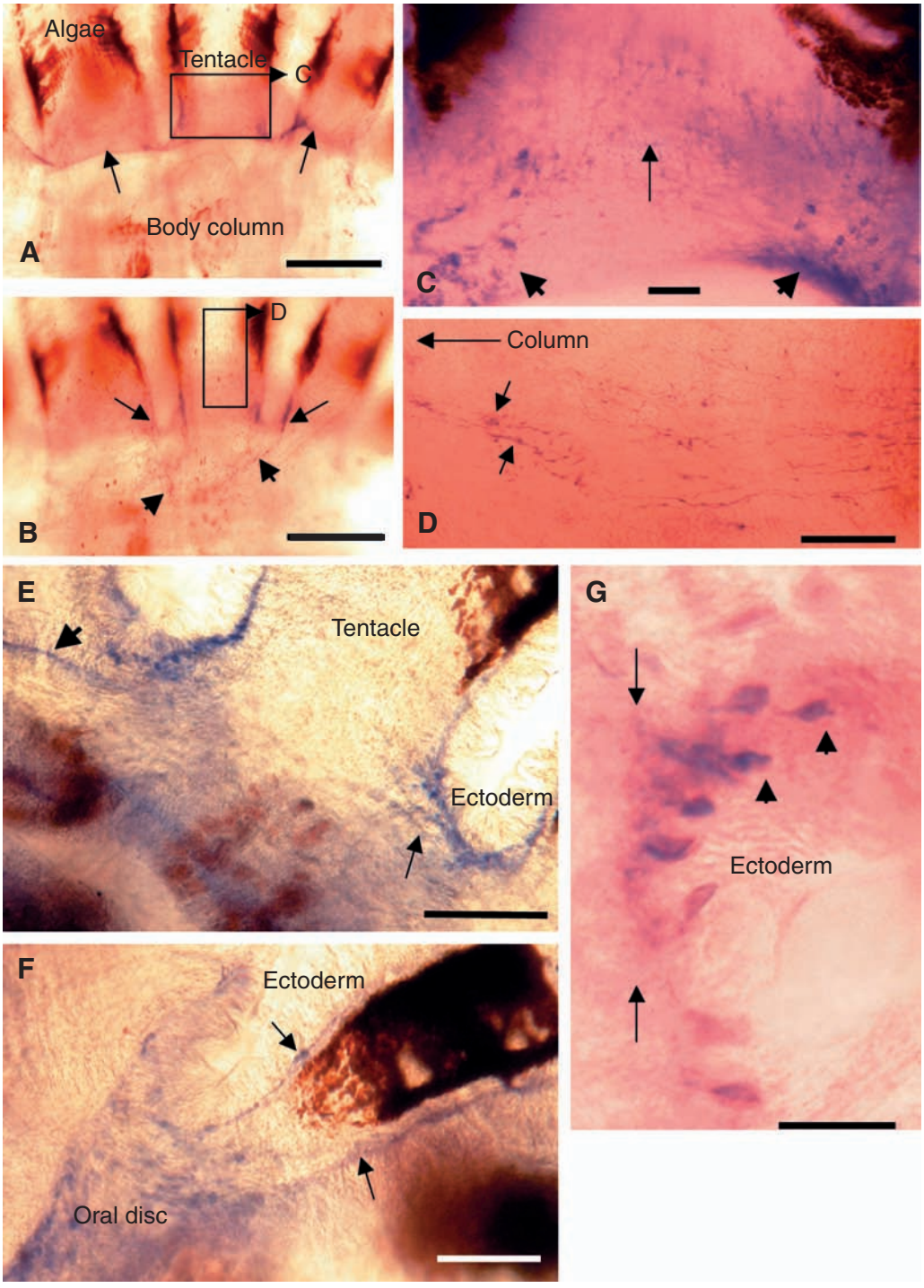

Fig. 8. Whole mounts showing the distribution of NADPH diaphorase staining in autozooid polyps of *R. koellikeri*. (A,B) Overview at two different focal planes of the upper part of a polyp where the base of tentacles is heavily populated by brown endosymbiotic algae. Note that focus is on staining of the oral disc in A, and on aboral tentacle and body column staining in B where bundles of tentacle neurites (arrows) extend to the body column (arrowheads). (C) Enlarged view of an oral disc region corresponding to the rectangle in A. Note clusters of cell somata at the margin (arrowheads) and meshwork of cell processes throughout the oral disc (arrow). (D) Enlarged view of a tentacle area corresponding to the rectangle in B. Note neurites running parallel to each other and cell somata (arrows) at the tentacle/body column interface. (E) Two bands of basiectodermal stained cell somata flank a tentacle on the aboral side. Processes from these cell bands extend towards the endodermal layer of the tentacle (arrow) and in the septum endoderm of the column (arrowhead). (F) Bands of stained neurites on the oral side of a tentacle (arrows) appear to merge into the similarly stained oral disk nerve-net. (G) Enlarged view of stained basiectodermal cell somata with an apical process (arrowheads). Neuritic processes from these cells (between arrows) extend towards the ectoderm/mesoglea interface. Scale bars: 200 μm (A,B), 25 μm (C), 30 μm (D), 80 μm (E), 50 μm (F) and 15 μm (G).

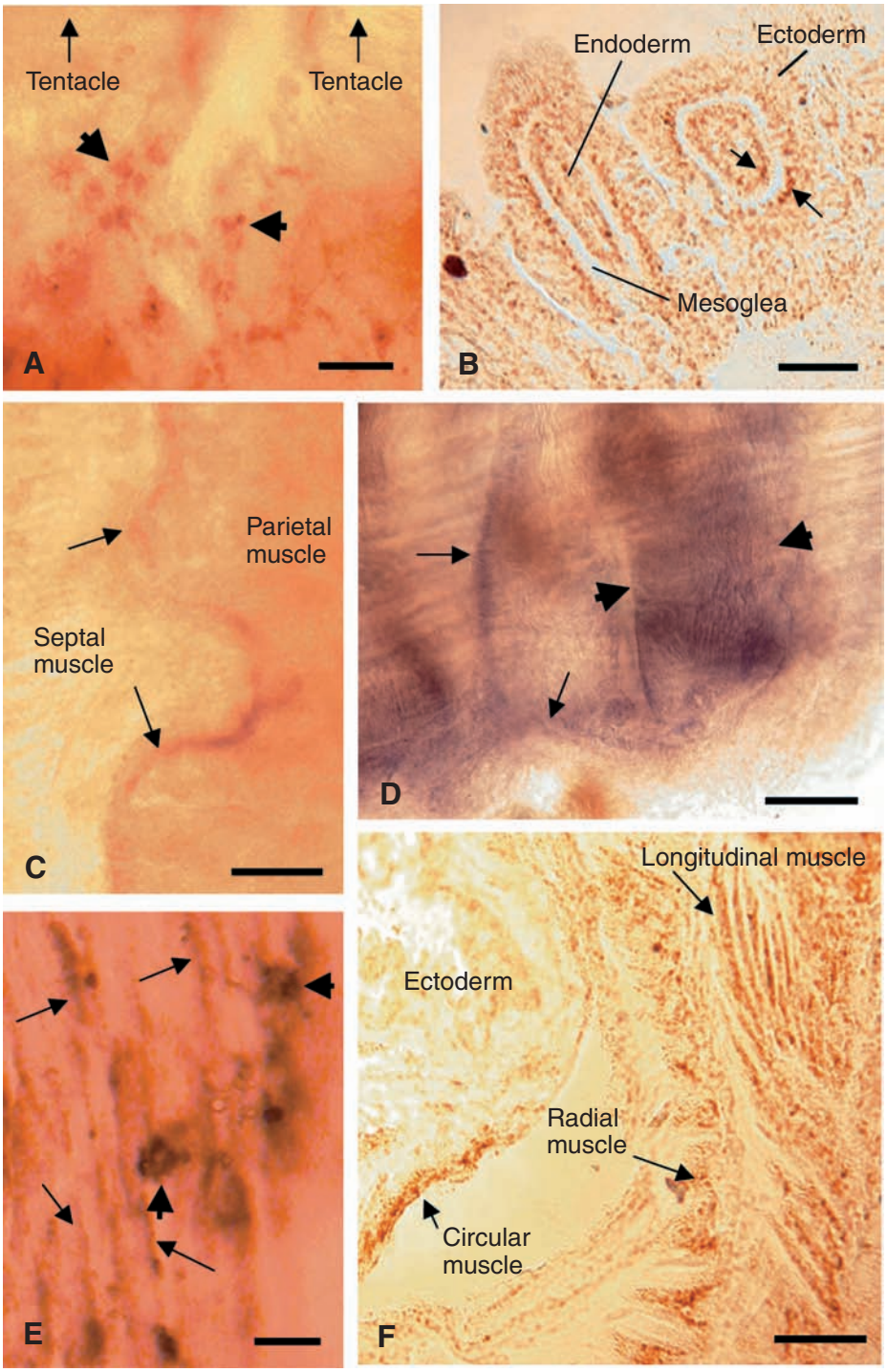

Fig. 9. NADPH diaphorase staining and citrulline immunostaining in the polyp anthocodium and the colonial mass. (A) Whole mount of tentacle/oral disk region showing citrulline immunoreactivity in basiectodermal cells (arrowheads). (B) Cross section through two tentacles showing bands of citrulline immunoreactivity at the ectoderm/mesoglea and at the mesoglea/endoderm boundaries (arrows). (C) Citrulline immunoreactivity (arrows) at the interface of body wall (parietal) and septal muscle in a whole-mount of polyp tissue. (D) Whole mount of lower polyp body column displaying NADPH diaphorase staining in circular muscle of the body wall (arrows) and in the muscle sheet of a flattened septum (between arrowheads). (E) Whole mount of circular muscle sheet inside the colonial mass in which both multipolar cells (arrowheads) and muscle feet (arrows) show citrulline immunoreactivity. (F) Section through the colonial mass in which are embedded the endodermal muscles of the submerged part (zooecium) of the polyps. Note the citrulline immunostaining in the septal (radial and longitudinal) muscles and in the circular muscle. Scale bars: 30 μm (A,C), 50 μm (B), 100 μm (D), 10 μm (E) and 40 μm (F).

known to be an effective and specific blocker of the serotonin response (Anctil, 1989), slightly reduced the potentiating effect of the NO donor SNAP on peristalsis (Fig. 3A), thus making it unlikely that serotonin has a major role in mediating the NO response.

Because the effect of aminoguanidine on peristalsis suggested the presence of an inhibitory nitrergic pathway, the effect of this iNOS blocker on the peristalsis-reducing response to melatonin was investigated. Aminoguanidine was unable to counteract the melatonin response (Fig. 5A), thus suggesting that NO is unlikely to mediate the melatonin response. However, melatonin appears to have prevented aminoguanidine from producing its potentiating effect on peristalsis, thus pointing to a pathway in which melatonin signalling would be downstream from iNOS-linked nitrergic signalling. In contrast, aminoguanidine was able to reverse the GnRH effect (Fig. 5A), but the reversal is partial and the amplitude of the GnRH response accounts for the difference between the partial reversal in the presence of aminoguanidine and the full potentiating response to aminoguanidine alone. Therefore it is likely that the inhibitory nitrergic and GnRH signalling pathways are independently activated.

Transduction of nitrergic signalling

NO action is usually mediated by the second messenger cGMP generated by the catalytic activity of a soluble form of guanylyl cyclase (Murad, 1996). A previous study showed that membrane-permeable analogues of cGMP mimicked melatonin in sharply reducing the amplitude of peristaltic

waves of the sea pansy (Anctil et al., 1991). Therefore it is not surprising that the data presented here suggest that the nitrergic pathway associated with reduction of peristalsis is mediated by cGMP. This is supported by the potentiating effect of the inhibitor of soluble guanylyl cyclase ODQ on peristalsis (Fig. 7A). This potentiating effect suggests that removal of cGMP from the tissues not only eliminates the inhibitory effect on peristalsis, but somehow also activates a potentiating pathway, possibly via cAMP, which potentiates peristalsis and has its levels raised by cGMP-lowering agents (Anctil, 1989; Anctil et al., 1991). As NO enhances peristalsis, it is not surprising that it has an additive effect to that of ODQ (Fig. 7A). In addition, the reduction of the melatonin response by half in the presence of ODQ also alludes to some form of interaction between melatonin and nitrergic signalling in inducing cGMP transduction.

The data suggest that two distinct forms of phosphodiesterase responsible for hydrolysing cGMP exist in the sea pansy. While zaprinast, a known selective inhibitor of cGMP phosphodiesterase (PDE5), decreased the amplitude of peristaltic waves by 30%, adding IBMX, an inhibitor of cAMP phosphodiesterase, had a greater, additive effect that almost obliterated peristalsis. As adding dibutyryl cGMP consecutively to zaprinast has nearly the same additive effect as IBMX, it is likely that IBMX acts by raising cGMP levels in the tissues. Anctil et al. (1991) have reported that in the sea pansy IBMX raises cGMP levels significantly while depressing cAMP levels. Although the functional significance of this finding is not yet clear and sea pansy phosphodiesterases remain to be characterized, the data point to the coexistence of zaprinast-sensitive and IBMX-sensitive phosphodiesterases, possibly having distinct functional roles. The existence of multiple isoforms of cGMP phosphodiesterase serving different roles in mammalian vascular smooth muscle is well documented (Beavo, 1995; Rybalkin et al., 2003).

Histochemistry of putative NOS-containing cells

The effects of NO donors and of NOS inhibitors on peristalsis indicated that NOS should be associated with endodermal muscles which cover the gastrovascular cavities and participate in peristalsis as well as in setting basal tension of polyps and colonial mass. Although both NADPH-d activity and citrulline immunostaining were located in these muscles, considerable staining was also present in basiectodermal neurons at the base of tentacles and in the oral disc of autozooid polyps. Staining specificity was ascertained by verifying that the NADPH-dependent NOS inhibitor diphenyleneiodonium abolished NADPH-d staining and that the NOS inhibitor 7-nitroindazole abolished citrulline immunostaining. Because there is no available NOS antibody that reacts with cnidarian tissues, NADPH-d staining has proved to be a reliable marker for multiple isoforms of NOS activity, and the case for its use with cnidarians has been persuasively made (Morrall et al., 2000; Moroz et al., 2004). Although this is the first report of citrulline immunoreactivity in a cnidarian, it is also considered a reliable marker of NOS activity in as much as controls include specific NOS inhibitors because of other potential metabolic sources of citrulline such as the urea cycle (Keilhoff and Wolf, 2003). The similar localization of these two different markers in sea pansy tissues is unlikely to be merely coincidental, which also argues for staining specificity. In the following analysis no distinction will be drawn between the two types of staining unless otherwise indicated.

The distribution of stained cells at the base of tentacles and in the oral disc, and of their accompanying neurites forming a nerve net, resembles the arrangement of the nitrergic neurosensory system described in the tentacles and bell margin of the jellyfish *Aglantha digitale* (Moroz et al., 2004). As in *A. digitale*, the stained cell somata of the sea pansy are located in the ectoderm, their neurites run up the tentacles and connect from the aboral to the oral side. However, the stained sea pansy neurons differ from those of *A. digitale* in that the cell somata are restricted to the base of tentacles and in the oral disc, and only their neurites run up to the distal tip of the tentacles. In addition, contrary to *A. digitale*, the stained cell somata are embedded at the base of the ectoderm, away from the external surface. Nevertheless, the association of NOS with the nervous system of an anthozoan as well as a hydrozoan indicates that nitrergic neurons with conserved distributions are a general feature of cnidarians.

A departure of importance from the nitrergic system of *A. digitale* is the localization of staining in the endoderm. In sea pansy whole-mount preparations, tracts of neurites were observed to run from the ectoderm across the mesoglea and into the endoderm of either tentacles or column of polyps. The presence of staining in the ectoderm and endoderm immediately adjacent to the mesoglea in cross sections of tentacles is consistent with this observation and with a similar distribution of NADPH-d staining in the column of the sea anemone *Aiptasia pallida* (Morrall et al., 2000). Thus, even though the endodermal muscles may lie within the range of action of NO released from the ectoderm across the thin mesoglea, according to calculations by Moroz et al. (2004), the possibility exists for NO to be released into endodermal muscles from local neurites. Moreover, the observation of citrulline-immunoreactive multipolar neurons in endodermal muscle sheets of the anthocodium and zooecium, with short neurites extending to muscle feet, suggests the existence of a second, entirely endodermal, neuromuscular nitrergic system in the sea pansy.

Assuming that the stained tentacle cells of the sea pansy are sensory, their deep location in the ectodermal epithelium would suggest that only a proprioceptive function is available to them. If so, one role of these nitrergic neurons could be to detect distortion from moving internal fluids by way of stretch receptors and to transmit output signals to other neurons or musculo-epithelial cells thereby directly adjusting muscle tension levels and peristalsis. This postulated mechanism has some analogy with the shearing forces acting on the luminal surface of mammalian vascular endothelium to regulate the release of NO (Buga et al., 1991; Smiesko et al., 1989). The location of these neurons in the tentacles and oral disc also

suggest a role in feeding, but preliminary experiments with NO donors and NOS inhibitors failed to affect the posture of tentacles or mouth opening. Also, the photocytes responsible for the sea pansy's bioluminescence are located in the endoderm just across the mesoglea from the stained neurons at the base of the tentacles. However, in our hands, NO donors had neither inducing effect on bioluminescence nor modulatory effect on electrically stimulated bioluminescence. Because the neurites of the tentacle cells do not appear to reach the lower part of the anthocodium and the zooecium where the bulk of muscles participating in peristalsis are located, the local endodermal multipolar neurons there could play a proprioceptive and modulatory role in colony-wide modulation of peristalsis and contracted state.

Functional significance

The data point to a complex involvement of NO signalling in setting basal muscle tension and peristaltic force in the sea pansy in response to putative interoceptive inputs. This could be achieved through two distinct nitrergic pathways, one responsible for muscle relaxation and lowered peristaltic force involving iNOS and soluble guanylyl cyclase, and the other associated with elevation of basal muscle tension and peristaltic force via another NOS isoform and possibly cAMP. The experimental evidence presented here indicates that these putative pathways do interact with indolaminergic transmitters, serotonin and melatonin, which themselves display opposite actions on peristaltic force. Ectodermal serotoninergic and melatoninergic neurons are distributed throughout the tentacles and column of the autozooid polyps and they possess long neurites like those of the putative nitrergic neurons (Umbriaco et al., 1990; Mechawar and Anctil, 1997), so the possibility exists of physical contacts between these three sets of neurons. The indolaminergic neurons differ from the putative nitrergic neurons in that the apical process of their somata reaches the external surface of the ectoderm. The serotoninergic neurons seem to detect the speed of water flow over the ectoderm surface and to mediate a peristalsis-adjusting response (Anctil, 1989; Umbriaco et al., 1990). Thus indolaminergic and nitrergic neurons may respond to different sensory modalities (exteroceptive and interoceptive, respectively) but interact with each other to modulate peristaltic force. The precise nature of these interactions and how they compare with the mammalian vascular model, in which sympathetic nerve stimulation causing vasoconstriction-derived shear leads to NO release and NO-mediated inhibition of norepinephrine release (Macedo and Lautt, 1996), need to be examined.

It is assumed that the two hypothesized nitrergic pathways in the sea pansy, each involving its own NOS isoform, would be present in different cell types, in accordance with the mammalian vascular model in which constitutive NOS is detected in endothelial cells and inducible NOS in smooth muscle cells (Cosentino and Lüscher, 1996). This cannot be demonstrated at this time because of the lack of reactivity of mammalian NOS antibodies with cnidarian NOS-like proteins. Nevertheless, the presence of NADPH-d and citrulline staining in ectodermal neurons forming a nerve-net strongly suggests the involvement of a neuronal form of constitutive NOS in these cells. Deriving the cellular origin of the putative iNOS is less straightforward, but endodermal musculo-epithelial cells are possible candidates even though it proved difficult to distinguish neuronal from musculo-epithelial staining in this layer. Because muscle oxidative enzyme activity unrelated to NOS may be revealed by NADPH-d staining (Planitzer et al., 2000), part of the NADPH-d (but not citrulline) staining appearing in sea pansy musculo-epithelial cells may not reflect the presence of NOS. Although much remains to be resolved at the cellular level, the present data point to the existence of two nitrergic pathways with distinct locations in the tissue layers of the sea pansy.

This study has demonstrated that NO can modulate two physiological parameters, basal tension and peristalsis, that affect the movement of fluid and food particles in the coelenteron of the sea pansy. The analogy with the NO-mediated modulation of vascular tone (Cosentino and Lüscher, 1996) and of peristalsis (Anand and Paterson, 1994) in mammals suggests that these physiological roles of NO were conserved throughout the evolution of metazoans. While other roles of NO were proposed for cnidarians, such as in the feeding behaviour of *Hydra* (Colasanti et al., 1997), the nematocyst discharge of a sea anemone (Salleo et al., 1996) and the swimming behaviour of a jellyfish (Moroz et al., 2004), this is the first report of NO involvement in cnidarian activities related to internal fluid movement.

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References

Anand, N. and Paterson, W. J. (1994). Role of nitric oxide in esophageal peristalsis. *Am. J. Physiol.* **266**, G123-G131.

Anctil, M. (1989). Modulation of a rhythmic activity by serotonin via cyclic AMP in the coelenterate *Renilla köllikeri*. *J. Comp. Physiol.* B **159**, 491-500.

Anctil, M. (2000). Evidence for gonadotropin-releasing hormone-like peptides in a cnidarian nervous system. *Gen. Comp. Endocrinol.* **119**, 317-328.

Anctil, M. and Poulain, I. (2003). Evidence of nitric oxide involvement in muscle systems of the octocorallian *Renilla koellikeri*. *Comp. Biochem. Physiol.* Part A **134**, S51.

Anctil, M., Pani, A. K. and Ali, M. A. (1991). Modulation of rhythmic contractions by melatonin via cyclic GMP in the coelenterate *Renilla koellikeri*. *J. Comp. Physiol. B* **161**, 569-575.

Beavo, J. A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* **75**, 725-748.

Buga, G. M., Gold, M. E., Fukuto, J. M. and Ignarro, L. J. (1991). Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension* **17**, 187-193.

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. (1998). Nitric oxide in invertebrates. *Mol. Neurobiol.* **17**, 157-174.

Colasanti, M., Venturini, G., Merante, A., Musci, A. and Lauro, G. M. (1997). Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *J. Neurosci.* **17**, 493-499.

Cole, A. G., Mashkournia, A., Parries, S. C. and Goldberg, J. I. (2002).

Regulation of early embryonic behavior by nitric oxide in the pond snail *Helisoma trivolvis*. *J. Exp. Biol.* **205**, 3143-3152.

Cosentino, F. and Lüscher, T. F. (1996). Nitric oxide and endothelial regulation of vascular tone. In *Methods in Neuroscience, Vol. 31: Nitric Oxide Synthase: Characterization and Functional Analysis* (ed M. D. Maines), pp. 215-227. San Diego, CA, USA: Academic Press.

Eliasson, M. J. L., Blackshaw, S., Schell, M. J. and Snyder, S. H. (1997). Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc. Natl. Acad. Sci. USA* **94**, 3396-3401.

Elofsson, R., Carlberg, M., Moroz, L., Nezlin, L. and Sakharov, D. (1993). Is nitric oxide (NO). produced in invertebrate neurones? *Neuroreport* **4**, 279-282.

Elphick, M. R. and Melarange, R. (1998). Nitric oxide function in an echinoderm. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **194**, 260-266.

Elphick, M. R., Kemenes, G., Staras, K. and O'Shea, M. (1995). Behavioral role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* **15**, 7653-7664.

Fautin, D. G. and Mariscal, R. N. (1991). Cnidaria: Anthozoa. In *Microscopic Anatomy of Invertebrates, Vol. 2: Placozoa, Porifera, Cnidaria and Ctenophora* (ed. F. W. Harrison and J. A. Westfall), pp. 267-358. New York, USA: Wiley-Liss.

Feelisch, M., Ostrowski, J. and Noack, E. (1989). On the mechanism of NO release from sydnonimines. *J. Cardiovascular Pharmacol.* **14**, S13-S22.

Gelperin, A., Flores, J., Raccuia-Behling, F. and Cooke, I. R. C. (2000). Nitric oxide and carbon monoxide modulates oscillations of olfactory interneurons in a terrestrial mollusc. *J. Neurophysiol.* **83**, 116-127.

Griffin, O. W. and Stuehr, D. J. (1995). Nitric oxide synthases: properties and catalytic mechanism. *Ann. Rev. Physiol.* **57**, 707-736.

Griffiths, M. J. D., Messent, M., McAllister, R. J. and Evans, T. W. (1993). Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br. J. Pharmacol.* **110**, 963-968.

Hogg, N., Darley-Usmar, V. M., Wilson, M. T. and Moncada, S. (1992). Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.* **281**, 419-424.

Jacklet, J. W. (1997). Nitric oxide signaling in invertebrates. *Invert. Neurosci.* **3**, 1-14.

Joly, G. A., Ayres, M., Chelly, F. and Kilbourn, R. G. (1994). Effects of N-methyl-L-arginine, N-nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun*. **199**, 147-154.

Keilhoff, G. and Wolf, G. (2003). Citrulline immunohistochemistry may not necessarily identify nitric oxide synthase activity: the pitfall of peptidylarginine deiminase. *Nitric Oxide* **8**, 31-38.

Lyke, E. B. (1965). The histology of the sea pansies, *Renilla reniformis* (Pallas) and *Renilla köllikeri* (Pfeffer) with a note on the fine structure of the latter species. PhD thesis, University of Wisconsin, Madison.

Macedo, M. P. and Lautt, W. W. (1996). Shear-induced modulation by nitric oxide of sympathetic nerves in the superior mesenteric artery. *Can. J. Physiol. Pharmacol.* **74**, 692-700.

Mechawar, N. and Anctil, M. (1997). Melatonin in a primitive metazoan: seasonal changes of levels and immunohistochemical visualization in neurons. *J. Comp. Neurol.* **387**, 243-254.

Melarange, R. and Elphick, M. R. (2003). Comparative analysis of nitric oxide and SALMFamide neuropeptides as general muscle relaxants in starfish. *J. Exp. Biol.* **206**, 893-899.

Moroz, L. L. (2000). On the origin and early evolution of neuronal NO signaling: a comparative analysis. In *Nitric Oxide and Free Radicals in Peripheral Neurotransmission* (ed. S. Kalsner), pp. 1-34. New York, USA: Springer-Verlag.

Moroz, L. L. (2001). Gaseous transmission across time and species. *Amer. Zool.* **41**, 304-320.

Moroz, L. L., Park, J. H. and Winlow, W. (1993). Nitric oxide activates buccal motor patterns in *Lymnaea stagnalis*. *Neuroreport* **4**, 643-646.

Moroz, L. L., Meech, R. W., Sweedler, J. V. and Mackie, G. O. (2004). Nitric oxide regulates swimming in the jellyfish *Aglantha digitale*. *J. Comp. Neurol.* **471**, 26-36.

Morrall, C. E., Galloway, T. S., Trapido-Rosenthal, H. G. and Depledge, M. H. (2000). Characterization of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Comp. Biochem. Physiol. Part B* **125**, 483-491.

Muller, U. (1997). The nitric oxide system in insects. *Progr. Neurobiol.* **51**, 363-381.

Murad, F. (1996). Signal transduction using nitric oxide and cyclic guanosine monophosphate. *J. Am. Med. Assoc.* **276**, 1189-1192.

Parker, G. H. (1920). Activities of colonial animals. I. Circulation of water in *Renilla*. *J. Exp. Zool.* **31**, 343-368.

Planitzer, G., Baum, O. and Gossrau, R. (2000). Skeletal muscle fibers show NADPH diaphorase activity associated with mitochondria, the sarcoplasmic reticulum and the NOS-1-containing sarcolemma. *Histochem. J.* **32**, 303-312.

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

Rybalkin, S. D., Yan, C., Bornfeldt, K. E. and Beavo, J. A. (2003). Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circulation Res*. **93**, 280-291.

Salleo, A., Musci, G., Barra, P. F. A. and Calabrese, L. (1996). The discharge mechanism of acontial nematocytes involves the release of nitric oxide. *J. Exp. Biol.* **199**, 1261-1267.

Schipp, R. and Gebauer, **M.** (1999). Nitric oxide: a vasodilatatory mediator in the cephalic aorta of *Sepia officinalis* (L.) (Cephalopoda). *Invert. Neurosci.* **4**, 9-15.

Scholz, N. L., Chang, E. S., Graubard, K. and Truman, J. W. (1998). The NO/cGMP signaling 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.

Smiesko, V., Lang, D. J. and Johnson, P. C. (1989). Dilator response of rat mesenteric arcading arterioles to increased blood flow velocity. *Am. J. Physiol.* **257**, H1958-H1965.

Trapido-Rosenthal, H. G., Sharp, K. H., Galloway, T. S. and Morrall, C. E. (2001). Nitric oxide and cnidarian-dinoflagellate symbioses: pieces of a puzzle. *Amer. Zool.* **41**, 247-257.

Trimmer, B. A., Aprille, J. R., Dudzinski, D. M., Lagace, C. J., Lewis, S. M., Michel, T., Qazi, S. and Zayas, R. M. (2001). Nitric oxide and the control of firefly flashing. *Science* **292**, 2486-2488.

Truman, J. W., de Vente, 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.

Umbriaco, D., Anctil, M. and Descarries, L. (1990). Serotonin-immunoreactive neurons in the cnidarian *Renilla koellikeri*. *J. Comp. Neurol.* **291**,167-178.