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Nitric oxide in control of luminescence from hatchetfish (*Argyropelecus hemigymnus*) photophores

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Summary

Nitric oxide synthase-like immunoreactivity (NOS-LI IR) was detected by immunohistochemistry in ventral light organs of the mesopelagic fish, *Argyropelecus hemigymnus*. Strong NOS-LI IR was present in nerve fibres and in other cells central for production or modulation of light: immunoreactive fibres surrounded the photophores, and were also present in the filter area. Filter cells, particularly in the outer layers, showed strong IR throughout the cytoplasm. Pharmacological studies suggested that nitric oxide (NO) modulates adrenaline-stimulated light emission, and that the modulation is correlated to the ability of the light organ to respond to adrenaline. Adrenaline is known to produce two different types of light response in isolated photophores from *Argyropelecus*: a slow, long-lasting, high intensity response, or a fast and weak response of short duration. Incubation of photophores in the NO donors sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine prior to adrenaline stimulation reduced the intensity of the strong and long-lasting type of response, but had little or even a potentiating effect on the weakly responding photophores. Hydroxylamine, which is converted to NO if catalase activity is present in the tissue, reduced the duration and the intensity of the adrenaline response in all tested organs. The NOS-inhibitor L-thiocitrulline potentiated the adrenaline response in the weakly responding organs; the weaker the adrenaline effect, the stronger the potentiation caused by L-thiocitrulline. The strongly responding organs were instead inhibited by L-thiocitrulline. The results suggest that NO has an important role in the control of light emission from *Argyropelecus hemigymnus* photophores. The cGMP analogue dibutyryl cGMP, the guanylate cyclase inhibitor ODQ and the phosphodiesterase inhibitor pentoxiphylline had no effect, indicating that the NO effect does not involve cGMP.

Key words: *Argyropelecus hemigymnus,* bioluminescence, teleost fish, nitric oxide, nitric oxide synthase.

Introduction

Photophores isolated from the mesopelagic luminescent fish *Argyropelecus hemigymnus* respond to the application of adrenaline with light emission of extremely variable kinetics and amplitude. Originally, photophores were divided into two types: the first produces a low level of luminescence with a fast extinction rate, and the second shows a high luminescence with a slow extinction rate (Baguet and Marechal, 1978). Although successive pharmacological analyses attempted to provide evidence for the roles of several different neuromediators in the control of photophores, all the results to date support the hypothesis that the ventral serial photophores are innervated by adrenergic nerves controlling the light emission (Baguet et al., 1980; Baguet and Mallefet, 1985; Salpietro et al., 1998).

Nitric oxide (NO) is a freely diffusible unconventional neurotransmitter and neuromodulator molecule that is increasingly found to play an important role in several physiological systems, from invertebrates to mammals (Jacklet, 1997). Since the presence of an NO system has been described in several fish species (Schober et al., 1993; Olsson and Holmgren, 1997; Nilsson and Söderström, 1997; Cox et al., 2001), we investigated the possible role of NO as a neurotransmitter and/or modulator of the adrenergic control of bioluminescence, using isolated ventral photophores of *Argyropelecus hemigymnus*.

The results suggest that NO is produced in the photophores and is involved in modulation of the adrenergic control of *Argyropelecus* luminescence. It is hypothesized that the very variable capacity of photophores to produce and maintain the luminescence evoked by adrenaline is correlated to a large variability in endogenous nitric oxide synthase (NOS) activity in the isolated photophores.

Materials and methods

Collection of fish

Fresh specimens of hatchetfish *Argyropelecus hemigymnus* Cocco were either captured in a hand net from the shallow waters of the Strait of Messina or collected along the shores of the Sicilian coast near Torre di Faro between 05.00 h and 06.00 h (cf. Baguet and Maréchal, 1976). They were brought to the laboratory (Biologia Marina ed Ecologie Animale, Ganzirri, Sicily or Istituto Talassografico di Messina, Sicily) in cooled seawater from the Strait, stored in a refrigerator at 7°C and used the same day.

Immunohistochemistry

Photophores (Fig. 1) obtained from 10 individuals were used for immunohistochemistry. The tissues were fixed for 16–20 h in Zamboni's fixative [15% picric acid, 2% formaldehyde in phosphate buffered saline (PBS, 0.9% NaCl, pH 7.2)]. After repeated washing in 80% ethanol, the tissue was dehydrated (95%, 99.5% ethanol, 30 min each), treated with xylene (30 min) and rehydrated (99.5%, 95%, 80% and 50% ethanol, 30 min each) to PBS. The tissues were stored overnight in PBS with 30% sucrose before embedding in OCT (Sakura, Zoeterwude, The Netherlands) or Agarose (Sigma Chemical Company, St Louis, MO, USA) and quick frozen in isopentane chilled with liquid nitrogen. Frozen samples that were not cut immediately were stored at –40°C until use.

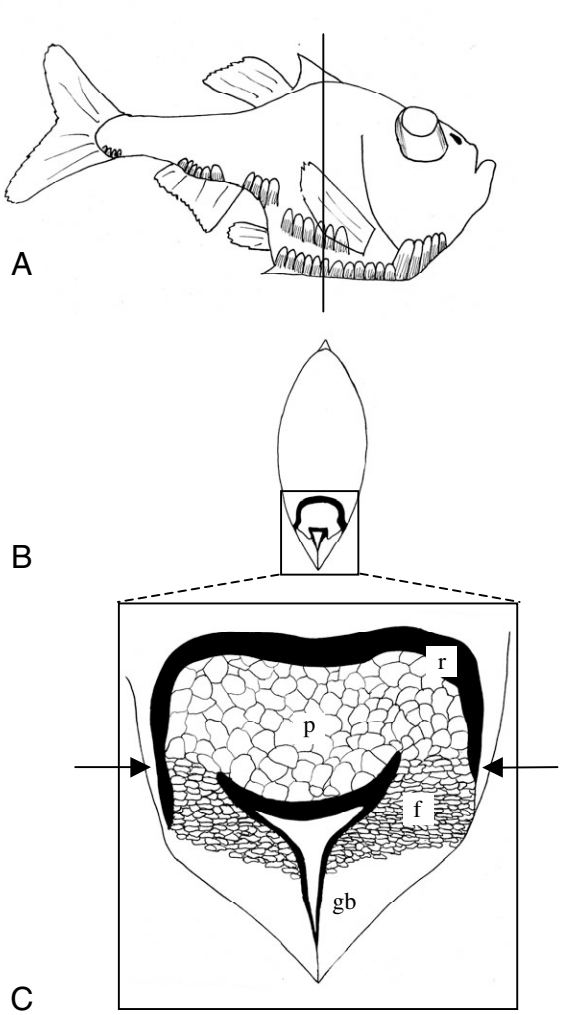

Fig. 1. Schematic drawing of the position (A,B) and structure (C) of the ventral photophores of the hatchetfish *Argyropelecus hemigymnus*. A common chamber for the photocytes (p) is surrounded by a reflector (r) (B,C). There are several exits for light from the photocyte chamber. The exits contain pigmented filter (f) cells and, more peripherally, unpigmented transparent tissue called the gelatinous body (gb; C) (Bassot, 1966; Denton et al., 1985). Arrows indicate the main entrance points for nerve fibres through the reflector to the photocyte chamber found in the present study (C).

A cryostat microtome (Zeiss Microm International GmbH, Walldorf, Germany) was used to cut 10 μm sections, which were captured on chrome alum gelatine-coated slides and left overnight to dry. The slides were stored at –20°C until use.

Fluorescence histochemistry

To prevent non-specific staining, sections were preincubated with normal donkey serum (10%) for 30–60 min. Primary antibodies (Table 1) were applied and the sections incubated for 48 h. They were rinsed (3× 5 min in PB with 2% NaCl) and incubated for 60 min with the secondary antibody (Table 1). All incubations were in a humid chamber at room temperature (20°C).

Rinsing was repeated as described above and the preparations were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) then examined using a Nikon eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DMX1200 digital camera. Captured images were processed in Adobe Photoshop.

Avidin–biotin histochemistry

The sections were preincubated with normal donkey serum as described above. Control tests with an additional preincubation with H_2O_2 (0.3 or 3% for 3 to 15 min) before the normal donkey serum incubation demonstrated no endogenous peroxidase activity. The primary and secondary antibodies (Table 1) were applied according to the description above. The sections were then incubated with avidin-biotinylated peroxidase complex (ABC Elite PK 6100 standard, Vector Laboratories) for 30 min and subsequently developed in Vector Nova red substrate kit (SK 4800, Vector Laboratories) for 3 min. The sections were mounted in 50% glycerol and 50% carbonate buffer (pH 8.5) and examined as described above.

Controls performed to confirm the specificity of the secondary antibodies, by omission of the primary antibody, did not reveal non-specific staining with any of the secondary antibodies in the test (Fig. 2). Absorption tests where the primary antibody nNOS SC1025 was preincubated overnight with excess of blocking peptide [the antigen, NOS1 (K-20) P sc-1025P] resulted in considerable quenching of the immunoreaction produced with that antibody (Fig. 3).

Table 1. *Antibodies and peptides used for immunohistochemistry*

Antibody/peptide	Host	Working dilution	Antigen (peptide sequence)	Source
Primary				
eNOS N30030	Rabbit	1:500	Human eNOS c-terminal (1030–1209)	Transduction Laboratories
iNOS N32030	Rabbit	1:1000	Mouse iNOS c-terminal (961–1144)	Transduction Laboratories
nNOS N31030	Rabbit	1:100	Human nNOS c-terminal (1095–1289)	Transduction Laboratories
nNOS SC1025	Rabbit	1:150	Human/mouse nNOS n-terminal	Santa Cruz Biotechnology
uNOS PA1-039	Rabbit	1:100	Murine i and nNOS c-terminal (1113–1122)	Affinity Bioreagents
Tubulin acetylated T-6793	Mouse	1:1000	Sea urchin acetylated tubulin	Sigma
Secondary				
DaM-biotin 715-065-151	Donkey	1:100		Jackson ImmunoResearch Laboratories
DaM-FITC 715-095-150	Donkey	1:100		Jackson ImmunoResearch Laboratories
DaR-biotin 711-065-152	Donkey	1:400		Jackson ImmunoResearch Laboratories
DaR-Cy3 711-165-152	Donkey	1:800		Jackson ImmunoResearch Laboratories
DaR-FITC 711-095-152	Donkey	1:100		Jackson ImmunoResearch Laboratories
Block peptide				
NOS1 (K-20) P sc-1025P		1:30		Santa Cruz Biotechnology

All antibodies against NOS are polyclonal. The antibody against acetylated tubulin is monoclonal.

DaM, donkey anti-mouse; DaR, donkey anti-rabbit; eNOS, enothelial nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; uNOS, universal NOS, i.e. antibody reacting to all NOS isoforms.

Pharmacology

Measurements of the light emission

The ventral photophores of *Argyropelecus* were dissected as described in Baguet and Marechal (1978). For each fish, the isolated ventral organ containing 24 photophores (Fig. 1) was split into an anterior and a posterior half, named 'anterior photophores' and 'posterior photophores', respectively.

The preparations were transferred to a small Perspex chamber and inserted into a slot (10 mm length × 2 mm wide) cut in the chamber; the light emitting area of the photophores facing a thin wall of Perspex orientated towards the photo detector of a luminometer (Berthold FB12; Pforzheim, Germany). Using the Berthold Multiple Kinetics program, it was possible to record simultaneously the luminescence from six preparations. The light sensitivity was calibrated using a standard light source (Betalight, Saunders Technology, peaking at 470 nm) positioned in place of the tissue. The response was recorded on a laptop computer. The following parameters were used to characterize the luminescence: the time (min) elapsed from the beginning of light emission to the time of maximum light emission (TL_{max}), the intensity (in Mq s^{-1}) of maximum light emission (L_{max}), and the time (min) to half-extinction of light emission ($TL_{1/2}$).

Drugs and solutions

The following chemicals were used in this study: adrenaline (Federa, Brussels, Belgium), dibutyryl-cGMP (Sigma), hydroxylamine hydrochloride (Acros organics, Pittsburgh, PA, USA), pentoxifylline (Sigma), (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); Tocris Cookson Inc, Ellisville, Missouri, USA), *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma), sodium nitroprusside (SNP; Sigma), L-thiocitrulline (Acros organics). All chemicals were dissolved just before use and diluted as required in a modified Hank's solution of the following composition (in mmol l^{-1}): NaCl 188, KCl 7.4, $CaCl_2{\cdot}2H_2O$ 3.5, $MgCl_2{\cdot}2H_2O$ 2.4, saccharose 120, adjusted to pH 7.3 with Tris-HCl buffer. Saccharose was added to raise the osmolarity to that of the blood serum of mesopelagic marine teleosts as measured by Griffith (1981).

Design of the experiments

To compare the effects of drug treatments on adrenaline-induced luminescence, six preparations (pairs of anterior and posterior halves of three ventral organs) were inserted in the slot of six Perspex chambers containing 500 µl Hank's solution and the zero level of light was controlled. For each pair of ventral organ halves, one was used as a control and maintained in saline, and the other was treated with a nitrergic drug, starting 20 min before light induction. For light induction, both control and treated photophores were stimulated with adrenaline at a final concentration of 10^{-4} mol l^{-1}. In one set of experiments with hydroxylamine, adrenaline stimulations were made first on both halves of the ventral organ, and the nitrergic drug was added to one half when maximal light production was reached. Anterior and posterior ventral organ halves isolated from different fish were used alternatively as control and treated preparations, in order to have the same number of control and treated preparations in the different pairs. The complete experimental procedure was performed at room temperature (18°C). Statistical analyses were performed using Student's *t*-test, methods of correlation and linear regression; each mean value is expressed with its standard error (mean ± S.E.M.) and (*N*) equals the number of preparations.

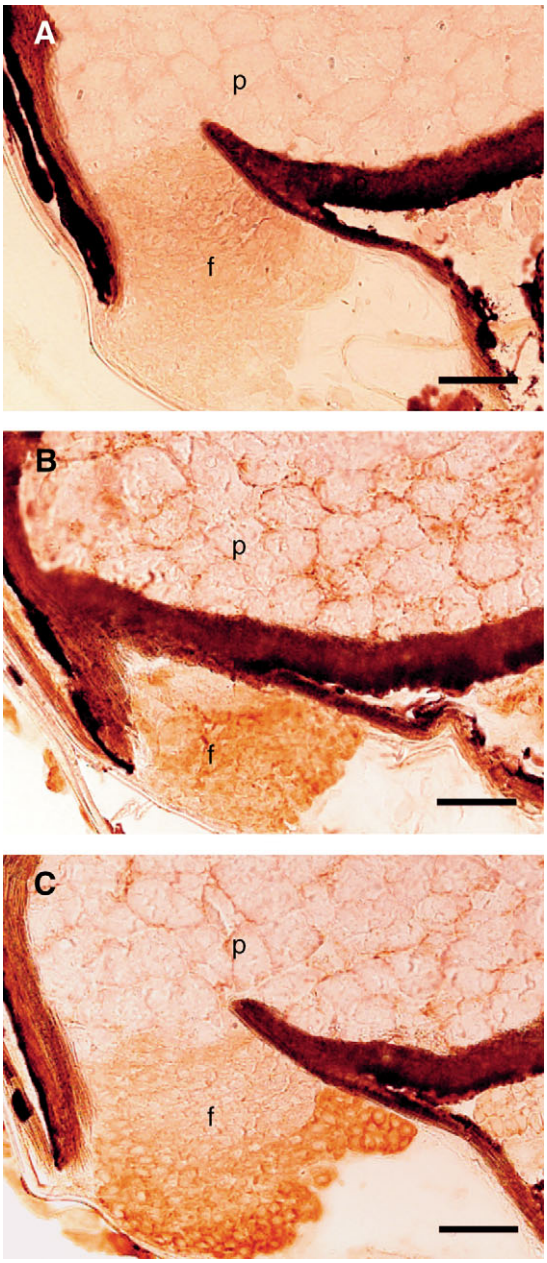

Fig. 2. Control experiments on sections of a ventral photophore from the hatchetfish *Argyropelecus hemigymnus*, showing (A) lack of unspecific staining after omission of primary antibody, and specific staining among the photocytes and in the filter cells, with (B) nNOS 31030 and (C) nNOS sc1025. f, filter cell area; p, photocytes. Bars, 50 µm.

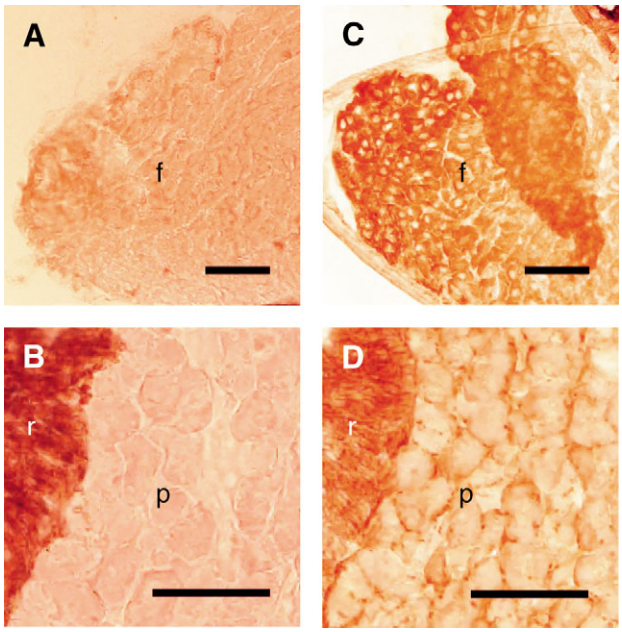

Fig. 3. Control experiments on sections of a ventral photophore from the hatchetfish, *Argyropelecus hemigymnus*. Preabsorption using antigen (block peptide, Table 1) and the antibody nNOS sc1025 (A and B) resulted in quenching of the immunoreaction both in the filter cells (compare A and C) and among the photocytes (compare B and D). f, filter cell area; p, photocytes; r, reflector. Bars, 50 µm.

Results

Immunohistochemistry

The structure of the *Argyropelecus* ventral organ with its 24 ventral photophores (Fig. 1) was described by Bassot (1966). Two or possibly three functional cell types were identified: (1) the photocytes enclosed in a common chamber delimited by a reflector, and (2) the B-cells or filter cells, which contain light absorbing pigment (Denton et al., 1985), and are located ventral to the photocytes (Fig. 4A). The cytological characteristics of the filter cells are gradually changing towards the outer part of the filter, possibly making the most peripheral (ventral) cells a third functional cell type (Bassot, 1966). The whole filter/B-cell area will from now on be referred to as the filter. This area is also commonly called the lens.

Antibodies raised against acetylated tubulin demonstrate the presence of nerve fibres in the filter area, as well as among the photocytes of *Argyropelecus* photophores (Fig. 4A,B). Nerve bundles of various diameters enter the photocyte chamber through the reflector layer at several locations and branch into thin fibre bundles or single fibres among the photocytes (Fig. 4B). In addition, fibres spread from a common point at the median narrowing of the photophore (where the reflector opens to the filter, Fig. 4C). The outer cell layers of the filter represent the most densely innervated area (Fig. 4A).

Nitric oxide synthase (NOS)-like immunoreactivity (NOS-LI IR) was frequently observed in nerve fibres, both in the nerve bundles leading into the photocyte chamber (Fig. 4E, compare also to Fig. 4B) and in thin varicose fibres running close to the photocytes (Fig. 4F,G). Positive NOS-LI IR was also observed in regularly distributed, unidentified intra- or extracellular structures closely associated with the plasma membrane of the photocytes (Fig. 4H).

Furthermore, NOS-LI IR was present within the cells of the filter. The immunoreaction was most intense in the outer cell layers, and showed a gradual reduction inwards through the

filter area (Fig. 4D). Sometimes only the outer cells contained NOS-LI IR. This gradient was most prominent when using the nNOS sc1025 antibody. Occasionally the most lateral cells of the filter appeared without staining in an otherwise strongly immunoreactive filter. Within the individual filter cells, NOS-LI IR was evenly distributed in the cytoplasm while the

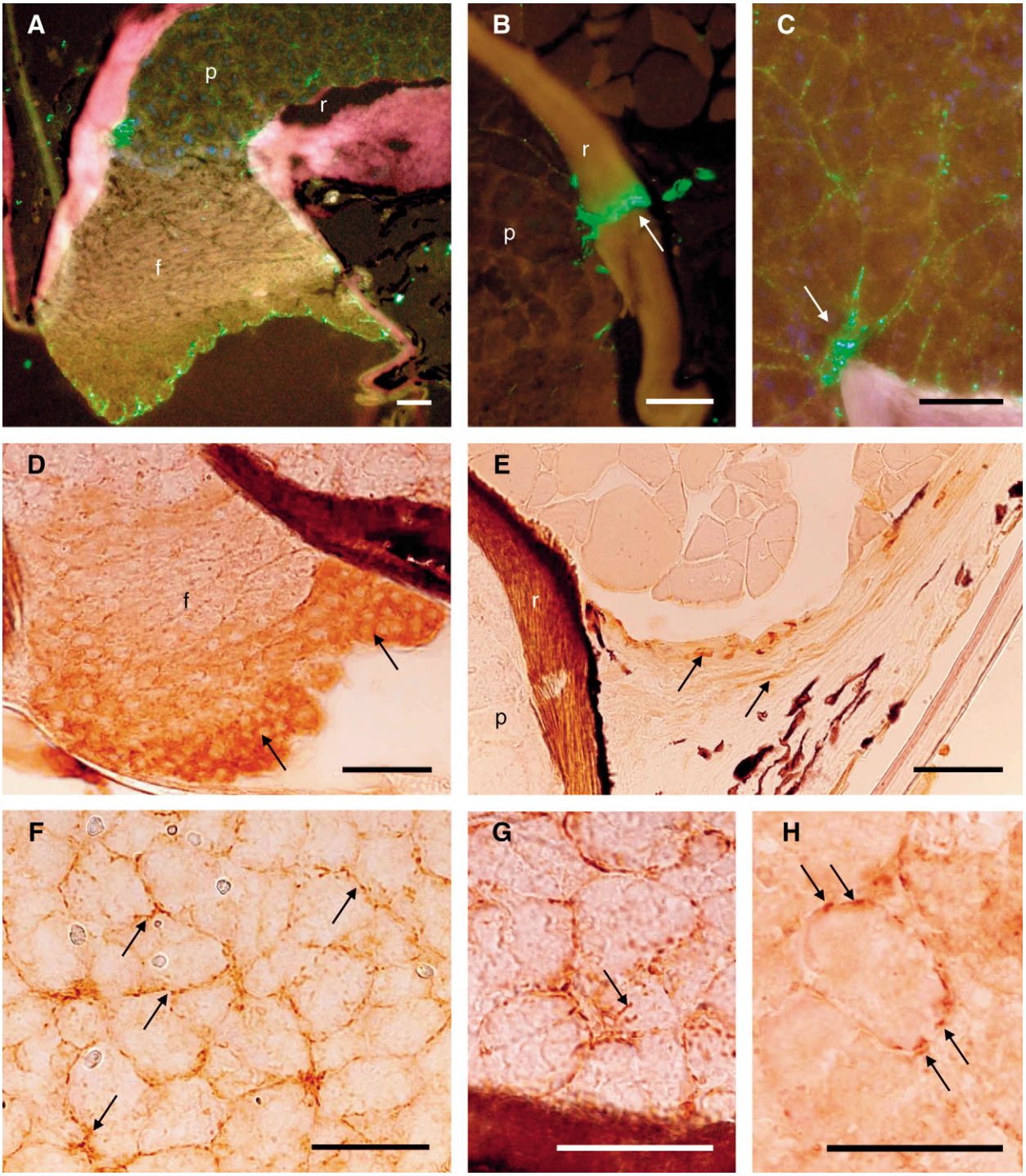

Fig. 4. Sections of ventral photophores from the hatchetfish, *Argyropelecus hemigymnus* showing acetylated tubulin-like (A–C; AcT-LI IR, green fluorescence) and nitric oxide synthase-like immunoreactivity (D–H; NOS-LI IR, brownish precipitate). Blue fluorescence in A–C is autofluorescence from the photocytes. (A) AcT-LI IR nerve fibres in the filter area, and among the photocytes. (B) Nerve bundle (arrow) entering the photocyte chamber through the reflector. (C) Nerves branching among the photocytes from a common point (arrow) at the median narrowing of the photophore. (D) NOS-LI IR (nNOS sc1025) in the cytoplasm of the outer filter cells (arrows). (E) NOS-LI IR (nNOS sc1025) in nerve bundle (arrows) leading to photophore. (F,G) Varicose nerve fibres (arrows) among photocytes showing NOS-LI IR (F, nNOS sc1025; G, nNOS 31030). (H) Intra- or extra-cellular structures with NOS-LI IR (arrows; nNOS 31030) at the cell membrane of a photocyte. f, filter cell area; p, photocytes; r, reflector. Bars, 50 µm.

nucleus and possibly the perinuclear space appear unstained. A few nerve fibres containing NOS-LI IR were seen among the filter cells situated close to the photocytes.

There appears to be a variation in NOS expression between individuals as well as within the same photophore. Variations were seen both in the labelling of the filter cell cytoplasm and in the presence of NOS-LI IR in nerves and other structures among the photocytes. In six out of ten individuals the immunoreactivity in structures among the photocytes was considerable, while it was hardly detectable in the remaining four. Weak NOS-LI IR among photocytes does not correlate with low immunoreactivity in the filter cells.

Antibodies against the three mammalian isoforms of NOS (neuronal NOS, nNOS; inducible NOS, iNOS; and endothelial NOS, eNOS) and one universal NOS (uNOS) antibody were used in the study (Table 1). Labelling varied slightly between different antibodies. Both antibodies against nNOS showed distinct nerve like structures among the photocytes. The N-terminal directed antibody nNOS sc1025 gave stronger labelling in the outer filter cells, while the C-terminal directed nNOS 31030 showed a more homogenous stain throughout the filter (this gradient is likely to be a fixation artefact, with the fixative affecting the N terminal more than the C terminal). The antibodies against iNOS and eNOS usually labelled the cytoplasm of the filter cells weaker than the antibodies against nNOS, and the labelling among the photocytes using these antibodies had a less distinct appearance. Furthermore, the antibody against iNOS gave a diffuse staining inside the photocytes. The universal antibody stained all structures weaker than the other antibodies used in the study.

Pharmacology

Control responses

Addition of adrenaline at a final concentration of 10^{-4} mol l^{-1} to control photophores always evoked a slow and long sustained emission of light. In this first series of experiments, the mean maximal value of the light response (L_{max}) measured on 21 preparations (184.80±96.71 Mq s^{-1}) was reached in 36.40±3.9 min (TL_{max}) and half-extinction ($TL_{1/2}$) occurred 43.78±6.27 min afterwards. The amplitude of the light response was extremely variable among preparations and was not correlated to the time to reach L_{max} (TL_{max}) or to the half-extinction time ($TL_{1/2}$). These parameters were not significantly different between the halves of the ventral light organ.

Effects of NO donors

Sodium nitroprusside (SNP)

The effects of the NO donor SNP were tested. Application of SNP at 10^{-3} mol l^{-1} for 20 min on isolated preparations did not induce light emission.

The effects of adrenaline were either enhanced or reduced after SNP treatment. One group of seven preparations showed a significant increase of the amplitude of the light emitted (+132.5±51.9 Mq s^{-1}). All individuals in this group had a low control value of L_{max} (47.2±16.7 Mq s^{-1}). The other group of

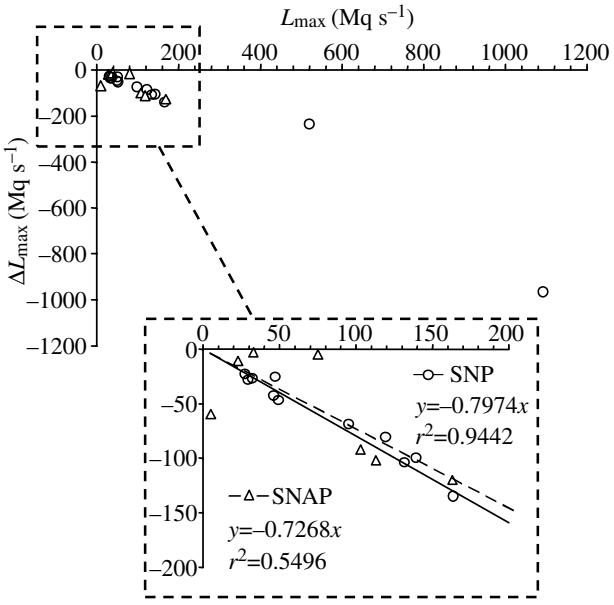

Fig. 5. SNP (circles) and SNAP (triangles) at 10^{-3} mol l^{-1} in 22 cases out of 34 decreased the adrenaline-induced luminescence of photophores. Insert, blow-up showing comparison of the relationship ΔL_{max}–L_{max} for lower L_{max} values. (The 12 instances where SNP or SNAP increased the adrenaline-induced luminescence are reported in the text.) The ordinate shows ΔL_{max} (differences between pairs of treated and control photophores in Mq s^{-1}); the abscissa, L_{max} (Mq s^{-1}) of the control photophores.

14 preparations showed a significant decrease of the light emission (–198.4±85.7 Mq s^{-1}). Individuals in this group had either a high or a low control value (Fig. 5).

A closer analysis of the data in the group where a decrease in light emission was obtained shows that the effect of SNP on the adrenergic response (i.e. the difference in L_{max} magnitude, ΔL_{max}) calculated for each pair of treated and control photophores, varies with the L_{max} value of the control preparation (Fig. 5). A regression line with a negative slope was calculated. These results suggest that the effect of SNP is not constant, but is dependent on the capacity of the photophore to luminesce in response to adrenaline. In contrast, the preparations reacting with an increased L_{max} after SNP treatment did not show any such correlation.

No significant correlation was observed between ΔTL_{max} and L_{max}, suggesting that SNP does not affect a mechanism controlling the rate of light production (Table 2). Similarly, extinction rate of the light emission does not seem to be affected by SNP since the mean $\Delta TL_{1/2}$ is not significantly different from control.

S-*nitroso*-N-*acetylpenicillamine (SNAP)*

In this second series of experiments, we studied the effects of SNAP, an NO source releasing a large proportion of NO oxidation products (Lemaire et al., 1999). We studied the effect of SNAP at 10^{-3} mol l^{-1} on the adrenergic response of 13 pairs of preparations.

Table 2. *Mean values of adrenergic light response parameters after treatments with NO donors as compared to control (Δ)*

	SNP (*N*=21)	SNAP (*N*=13)
ΔTL_{max} (min)	5.47±3.55	3.07±7.29
$\Delta TL_{1/2}$ (min)	–0.97±7.30	–19.23±4.60***

SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TL_{max}, time to maximal light emission; $TL_{1/2}$, time from maximal to half extinction of light emission.

***P<0.001.

Adrenaline controls: TL_{max}=36.40±3.9 min; $TL_{1/2}$=43.78±6.27 min (*N*=21).

20 min of SNAP application did not induce luminescence. As described for SNP, it was found that one group of preparations (*N*=5) showed a significant increase of L_{max} value (+32.85±11.78 Mq s^{-1}), while the other group (*N*=8) showed a significant decrease of L_{max} (–52.56±16.71 Mq s^{-1}). The differences (ΔL_{max}) vary as a function of L_{max} values of the control preparations: a highly significant correlation was calculated (Fig. 5). As the negative slope of the regression line is not different from that found for SNP, it is suggested that SNP and SNAP have similar effects on adrenaline-induced luminescence of photophores.

No significant difference in the effects of adrenaline on TL_{max} between control and treated preparations was obtained (Table 2), suggesting that SNAP does not affect the processes controlling the rate of light production. On the other hand, $\Delta TL_{1/2}$ is highly significantly negative, showing that SNAP accelerates the extinction rate. This effect of SNAP on the light extinction of treated preparations is correlated (P<0.001) to $TL_{1/2}$ of control preparations. In this case, a significant regression line with a negative slope (–0.60±0.12) could be calculated, indicating that SNAP accelerates the extinction rate more the slower the initial rate of light extinction.

Hydroxylamine

A series of eight light organs was used to study the effect of hydroxylamine, which is converted to NO when catalase activity is present in the treated organ (DeMaster et al., 1989). Among the eight hydroxylamine (10^{-3} mol l^{-1}) treated preparations, four preparations were completely inhibited when stimulated with adrenaline; the other four preparations showed a very feeble response with a mean amplitude reduced to 3.23±0.95% of the control (Fig. 6A).

Hydroxylamine added to luminescing photophores rapidly accelerated the extinction rate: Fig. 6B shows the mean value of the extinction time course calculated on eight pairs of adrenaline stimulated preparations: the time to reach 50% of L_{max} decreased from 7.0 min measured in control to 3.3 min after addition of hydroxylamine 10^{-3} mol l^{-1} (Fig. 6B).

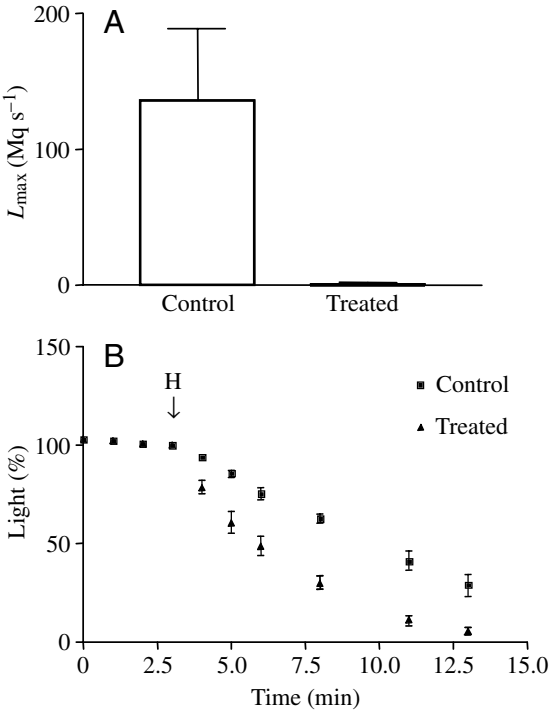

Fig. 6. (A) Comparison of the maximal amplitude of adrenergic response of 8 pairs of control and hydroxylamine (10^{-3} mol l^{-1}) treated photophores. (B) Extinction time course for luminescence from adrenaline stimulated photophores. On addition of hydroxylamine (H, arrow), treated photophores show an acceleration of light extinction as compared with control photophores.

Effects of NOS inhibitor

Although NO donors affect the light response to adrenaline, this finding cannot be used to assert that NO can be generated in the photophores and involved in the control of luminescence. To examine the potential endogenous generation of NO in the light emission of the light organs, we studied the effects of L-thiocitrulline, a potent inhibitor of both constitutive and inducible isoforms of NOS (Narayanan and Griffith, 1994) on the adrenergic response of photophores.

The effects of thicitrulline were examined using 13 pairs of control and treated preparations. 20 min pre-treatment with L-thiocitrulline at 10^{-3} mol l^{-1} failed to induce luminescence of isolated light organs, but affected the response to a subsequent exposure to adrenaline (Fig. 7). Fig. 7A shows that for the different pairs of photophores, the L_{max} change induced by L-thiocitrulline (ΔL_{max}) is related to L_{max} of the control photophore. The relationship is highly significant (P<0.002) with a negative slope (–1.02±0.18) for the calculated regression line. The analysis shows that ΔL_{max} is positive in the range of low L_{max} values; the lower the capacity of adrenaline to induce light on its own, the stronger the potentiation caused by L-thiocitrulline. Highly luminescing photophores can instead be predicted to be inhibited by NOS blockade.

A characteristic of the adrenergic light emission of control photophores is the absence of a correlation between the parameters of the light response. In treated preparations (Fig. 7B), we observed a significant correlation between TL_{max}

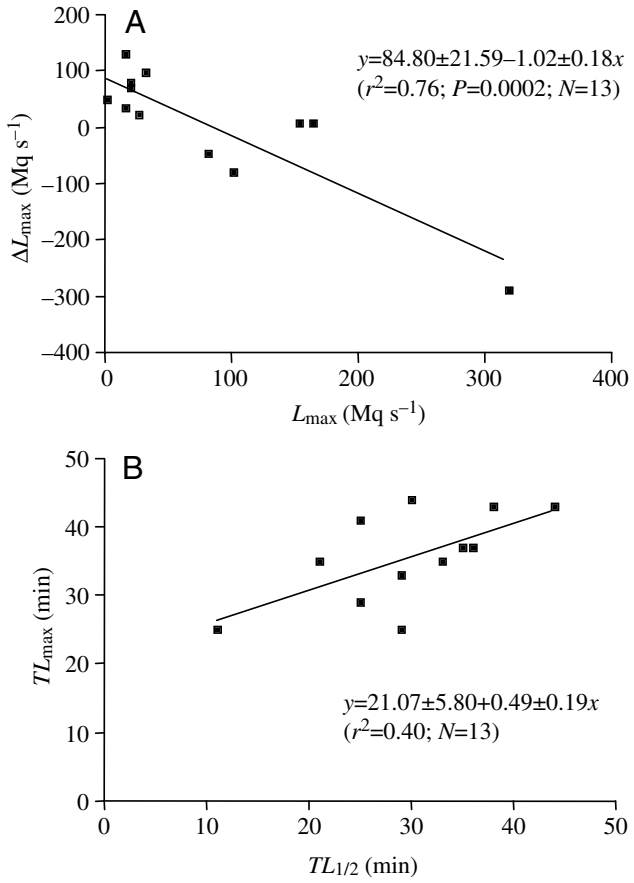

Fig. 7. (A) L_{max} differences (ΔL_{max}) between pairs of L-thiocitrulline treated and control photophores plotted in function of L_{max} (Mq s^{-1}) of control photophores. (B) Relationship between rates of light production (TL_{max}) and light extinction ($TL_{1/2}$, min) in L-thiocitrulline treated photophores.

and $TL_{1/2}$: the slope of the regression line (0.49±0.19; $P<0.002$) shows that the rate of light production varies in proportion to the rate of light extinction.

Effects on the cyclic GMP pathway

Since NO is the most potent and effective activator of soluble guanylate cyclase (Maréchal and Gailly, 1999), we investigated whether the effects of NO occur *via* the synthesis of cyclic guanosine monophosphate (cGMP), the possible second messenger between NO and luminescence inhibition.

To test this hypothesis, we first investigated the effects of dibutyrylguanosine 5′,5′-cyclic monophosphate (db-cGMP) a membrane-permeable analogue of cGMP, on the adrenergic light response in a series of 14 light organs. The application of db-cGMP at 10^{-3} mol l^{-1} for 20 min did not induce luminescence. The pattern of the adrenergic response was similar in both treated and control preparations and no significant differences were observed between TL_{max}, L_{max} and $TL_{1/2}$ values.

To test the presence of guanylate cyclase in the light organs and its possible activation by endogenous NO production, we examined the effects of ODQ, a potent and selective inhibitor of NO-sensitive guanylate cyclase (Garthwaite, 1995) in another series of six light organs.

During the 20 min treatment of the preparations with ODQ at 10^{-3} mol l^{-1}, no light emission was detected; the mean value of the parameters of the response to adrenaline (TL_{max}, L_{max} and $TL_{1/2}$) was similar in both treated and control preparations.

To test the possible rapid catabolism of endogenous cGMP by the catalytic activity of phosphodiesterase in the light organs, we studied the effects of pentoxiphylline, an inhibitor of phosphodiesterases. In this case, as in the case of db-cGMP and ODQ, we did not observe any significant effect on the adrenergic light response.

Discussion

The present study shows, for the first time, that NOS-like material is present in the photophores of a bioluminescent fish species, the hatchetfish *Argyropelecus hemigymnus*, and that several drugs acting on NO formation and function modulate adrenaline-induced light emission from isolated photophores.

Immunohistochemistry

NOS immunoreactivity was localised to several different structures: in nerves throughout the photophores, in plasma membrane-associated structures of photocytes, and apparently in the cytoplasm of the filter cells. This agrees with an involvement of NO in the control of light production and/or emission from the photophore. Varicose nerve fibres running close to the photocytes, and the plasma membrane-associated stores of NOS-LI IR material, may be directly involved in the control of light production, while nerve fibres innervating the filter area and cytoplasmic NOS in the filter cells may be involved in the control of light emission from the photophores.

The plasma membrane-associated stores of NOS might be vesicles or organelles. In neurons from the brain of the Atlantic salmon, nNOS-immunoreactivity has been detected in mitochondrial membranes as well as in vesicles (Holmqvist and Ekström, 1997). Furthermore eNOS-immunoreactivity has been found in mitochondria closely associated to the cell membrane in skeletal muscle from rat (Kobzik et al., 1995).

In this study, we have investigated the involvement of NOS/NO in adrenaline induced light production, but it is feasible that NO is involved in other, both general (metabolic) and specific, processes in the photophores. Our positive results, together with previous histochemical results in other non-mammalian vertebrates including teleosts (Holmqvist et al., 1994; Holmqvist and Ekström, 1997; Olsson and Holmgren, 1997; Karila and Holmgren, 1997; Funakoshi et al., 1999), suggest that NOS is a highly conserved enzyme amongst vertebrates. Several studies have established the presence of n-type NOS and i-type NOS, but not eNOS, in teleost fish (e.g. Laing et al., 1999; Øjan et al., 2000; Cox et al., 2001; Jennings, 2004). The antibodies used in the present study were raised against the mammalian isoforms of NOS (nNOS, eNOS,

iNOS). The strongest and most distinct staining was obtained with antibodies raised against the C-terminal or the N-terminal of nNOS, and occurred in nerve-like structures as well as in structures that are not expected to express or contain nNOS (filter cell cytoplasm, mitochondria-like structures). This suggests that the NOS isoform(s) present in the light organ of hatchetfish is/are more similar to mammalian nNOS than to mammalian eNOS or iNOS. A conclusive determination of the identity of NOS type(s) in the light organ needs further molecular study but is outside the aims of the present work.

Effects of NO donors

Our results show that all the tested NO donors (SNP, SNAP and hydroxylamine) affected, in a specific way, the amplitude of the light response evoked by adrenaline. SNAP and hydroxylamine in addition increased the rate of light extinction.

Hydroxylamine completely inhibits the light response of the isolated light organ, and induces a rapid extinction of luminescence induced by adrenaline. Hydroxylamine is a known precursor of nitric oxide in biological systems that exhibit a catalase activity (Keilin and Nicholls, 1958; DeMaster et al., 1989). Catalase, unlike superoxide dismutase and glutathione peroxidase, is present in mesopelagic fish tissues (Janssens et al., 2000). Our results suggest the presence of catalase in *Argyropelecus* light organs, and a synthesis of nitric oxide that should mediate the hydroxylamine-induced inhibition and extinction of luminescence. In this case, the generation of NO by endogenous catalase in the light organ seems to be a potent inhibitor of luminescence.

The inhibitory effect of the NO-donors SNP and SNAP is not constant but depends on the capacity of the preparation to luminescence: inhibition is maximal in light organs that produce a large adrenaline-induced luminescence, and it is minimal in those showing a low adrenergic response. The high variability of the inhibitory effect agrees with the very large variability of the NO synthase activity present in the photophores as revealed by immunohistochemistry.

The low adrenergic light emission of some light organs could be due to a high endogenous NOS activity; in this situation the contribution of an external source of NO (SNP or SNAP) might not cause an additional inhibitory effect. On the other hand, in light organs that show a large adrenergic response, the endogenous NOS activity might be too low to affect the light emission; in this case the external source of NO could exert an inhibitory effect.

SNAP and hydroxylamine, in addition, accelerated the rate of light extinction of the adrenergic response, as indicated by the reduced $TL_{1/2}$ value, while SNP apparently did not. A possible explanation for the differences in magnitude and character of the response is that the same concentration of NO donor may give different concentrations of NO as well as different proportions of NO *vs* oxidation products. Furthermore, different donors may produce NO in different tissue compartments (extracellular *vs* intracellular; Ioannidis et al., 1996; Ohta et al., 1997). Similarly, the fact that SNP in several cases increases, rather than decreases, the maximal light production appears to be anomalous. The mechanisms behind this need further investigation. Nevertheless, the increase in light production, when it occurs, is restricted to weakly luminescing photophores. Possibly, NO-production is used as a link in several, counteracting mechanisms balancing the output of light from the photophores in the countershading situation.

Effects of NOS inhibition

The effect of NOS-inhibition on maximal light production, with a large stimulation of weakly luminescing photophores and a weaker or no stimulation of some of the strongly luminescing photophores, is compatible with the view that the capacity of the photophores to respond to adrenaline is inversely proportional to the activity of (endogenous) NOS in the photophores. The fact that some highly luminescing photophores instead respond with a decrease in maximal intensity may appear more inexplicable, but supports the theory above that several mechanisms depending on NO production are involved in balancing the output from the light organ for countershading. Notably, the inhibition of NOS seems to synchronize kinetics of generation and extinction in photophore luminescence.

Similar multifaceted roles for NO, depending on the conditions and levels of its production, have been proposed in other physiological systems. NO production has been reported to improve mammalian heart efficiency (Bernstein et al., 1996), as well as the efficiency of skeletal muscle (Maréchal and Gailly, 1999). We could speculate that in the light organ, a minimal production of NO is essential to control the proportion of chemical energy effectively transformed into light. Clearly, further studies of the effects of NO on light organ metabolism are needed.

Mechanism of NO action

The physiological effects of NO in many tissues are mediated either by cGMP, formed by guanylate cyclase when stimulated by NO, or by modulation of mitochondrial respiration by competing with oxygen for cytochrome oxidase (Shen et al., 1994; Brown, 1995; Stamler et al., 1997). Since our results show that addition of exogenous db-cGMP, as well as inhibitors of c-GMP catabolism or NO-sensitive guanylate cyclase, have no effect on the adrenergic light emission, it is very likely that the NO-cGMP pathway is not important in the light production of the isolated light organs of *Argyropelecus hemigymnus*. Instead, we suggest that in *Argyropelecus* photophores, NO affects luminescence levels, by modulating mitochondrial respiration of the light organ. It is known that the capacity of isolated photophores to produce light increases in proportion to the intensity of the resting respiration rate measured before the adrenergic light response (Baguet and Mallefet, 2000) and that during light production, the oxygen consumption initially drops and then increases to a level slightly below the initial resting level (Mallefet and Baguet, 1985, 1991).

Concluding remarks

According to the countershading hypothesis, the photophores of *Argyropelecus* can luminesce continuously (Clarke, 1963), the intensity being rapidly adjusted and modulated to match that of the background twilight. Amongst all the neurotransmitters tested, adrenaline alone induces luminescence of *Argyropelecus* photophores *in vitro*, and it is generally accepted that an adrenergic stimulatory nervous system should be responsible for the tonic luminescence of the photophores. However, it is likely that some balancing inhibitory mechanism is necessary to perfectly match the background levels of light. Unfortunately, all the previously tested neurotransmitters failed to induce any extinction of the luminescence.

Based on the present results, we can now speculate that NO has a specific behavioural role to modulate the light emission of the luminescing photophore. By a reversible inhibition of mitochondrial respiration of the luminous cells, NO could rapidly change the luminescence level generated by the adrenergic nervous system. Furthermore, a large difference in NOS expression or activity among the different preparations may account for the well-known high variability in the amplitude and the extinction rate of the adrenergic light responses of light organs isolated from *Argyropelecus* (Baguet and Marechal, 1978).

List of abbreviations

AcT-LI IR	acetylated tubulin-like immunoreactivity
db-cGMP	dibutyrylguanosine 5′,5′-cyclic monophosphate
cGMP	cyclic guanosine monophosphate
ΔTL_{max}	TL_{max} (control preparations)–TL_{max} (treated preparations)
$\Delta TL_{1/2}$	$TL_{1/2}$ (control preparations)–$TL_{1/2}$ (treated preparations)
ΔL_{max}	L_{max} (control preparations)–L_{max} (treated preparations)
eNOS	endothelial nitric oxide synthase
eNOS-LI IR	endothelial nitric oxide synthase-like immunoreactivity
iNOS	inducible nitric oxide synthase
IR	immunoreactivity
L_{max}	maximal light emission
NO	nitric oxide
NOS	nitric oxide synthase
NOS-LI IR	nitric oxide synthase like immunoreactivity
nNOS	neuronal nitric oxide synthase
nNOS-LI IR	neuronal nitric oxide synthase-like immunoreactivity
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PBS	phosphate buffered saline
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNP	sodium nitroprusside
TL_{max}	time to maximal light emission
$TL_{1/2}$	time to half extinction of maximal light emission
uNOS	universal nitric oxide synthase

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