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Endothelin receptors in teleost fishes: cardiovascular effects and branchial distribution

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Stensløkken, Kåre-Olav, Lena Sundin, and Göran E. Nilsson. Endothelin receptors in teleost fishes: cardiovascular effects and branchial distribution. *Am J Physiol Regul Integr Comp Physiol* 290: R852–R860, 2006. First published October 13, 2005; doi:10.1152/ajpregu.00618.2004.—By observing gill blood flow using epi-illuminating microscopy, in parallel with cardiovascular recordings and immunohistochemistry, we have tried to identify the receptor mediating endothelin (ET) type 1 (ET_1)-induced pillar cell contraction in the lamellae of the Atlantic cod (*Gadus morhua*). Intra-arterial injection of the specific ET_B receptor agonist BQ-3020 induced dose-dependent increases in ventral aortic blood pressure, gill vascular resistance, and pillar cell area (indicating contraction). The specific ET_A receptor antagonist BQ-610 did not prevent either pillar cell contraction or increased gill vascular resistance induced by ET-1 injection. The cardiovascular responses were corroborated by the detection of ET_B receptor-like immunoreactivity (IR) associated with pillar cells in the lamellar region and in neuroendocrine cells. ET_B receptor-like IR was also found lining the muscle layer of lamellar arterioles and filament arteries. In contrast, strong ET_A receptor-like IR was found on branchial nerves throughout the filaments. In addition, ET-like IR was concentrated in neuroendocrine cells in the filament and lamellae. We also present data suggesting that ET-mediated pillar cell contraction is widespread among teleost fish, including Atlantic cod, rainbow trout (*Oncorhynchus mykiss*), sculpin (*Myoxocephalus scorpius*), and mackerel (*Scomber scombrus*). Taken together, our results suggest that an ET_B-like receptor mediates pillar cell contraction in fishes, whereas ET_A-like receptors may serve another function in the gill, inasmuch as ET_A receptor-like IR is found on branchial nerves.

gill; pillar cell; nerve; BQ-3020; BQ-610

SINCE THE DISCOVERY of the 21-amino acid peptide hormone endothelin (ET) type 1 (ET-1) by Yanagisawa et al. (39), many functions of this strong vasoconstrictor have been detected and described in mammals. Two other peptides in the family have also been discovered in mammals, ET-2 and ET-3, which differ from ET-1 in two and six amino acid residues, respectively. In addition, an ET purified from trout kidney (36) differs from human ET-2 in three and from human ET-1 in four amino acid residues. Curiously, homologous trout ET showed a weaker cardiovascular potency than ET-1 in rainbow trout (15). In mammals, two main subtypes of ET receptors have been described: an ET_A receptor, which mediates vasoconstriction in blood vessels, and an ET_B receptor, which probably facilitates the release of local endothelium-derived relaxing factors such as nitric oxide and prostacyclin (for review see Ref. 20). An ET_{B2} receptor, which constricts vascular smooth muscle, has also been described in mammals (37).

The first evidence for ET-like immunoreactivity (IR) in fish was reported in 1991, when immunolabeled axons were detected in the brain of medaka (*Oryzias latipes*) and IR cells were observed in its gills (18). Also in 1991, ET-1 was found to increase gill vascular resistance (R_{gill}) in perfused gills and to cause a transient, dose-dependent reduction in the dorsal aortic blood pressure (P_{DA}) in vivo in rainbow trout (*Oncorhynchus mykiss*) (25). Since then, ET-like IR has been found in neuroendocrine cells (NEC) in fish gills of several species (40), and ET receptors have been detected with autoradiography in rainbow trout gills (19). On the basis of the difference in affinity between the two receptor types for ET-1, ET-2, and ET-3 in mammals, Lodhi et al. (19) argued for an ET_A-type receptor in the branchial vasculature. The mammalian ET_A receptor binds ET-1 with a much higher affinity than ET-3, whereas the ET_B receptor has a similar affinity for ET-1 and ET-3. Lodhi et al. (19) found that ET-1 was much more effective than ET-3 in inhibiting binding of ^{125}I-labeled ET-1. On the other hand, in the spiny dogfish shark (*Squalus acanthias*), there is evidence for ET_B-like receptors in the gill (6) and aorta (5). In the eel (*Anguilla rostrata*), pharmacological evidence points toward involvement of an ET_B-like receptor in bulbus arteriosus contraction, but the presence of ET_A-like receptors could not be ruled out (8). Moreover, Evans and Harrie (7) showed that the ventral aorta of the eel does not respond to the specific ET_B receptor agonist SRX S6c. Thus it appears that ET_A and ET_B receptors can mediate vasoconstriction in fish vessels and that there may exist species and/or tissue differences with regard to the receptors involved.

An ability to regulate the functional respiratory surface area of the gills should be of fundamental importance for the respiratory and ion-regulatory physiology of fishes. This is because there is a trade-off between oxygen uptake and ion homeostasis (i.e., exposing the blood to hypo- or hyperosmotic water over a large respiratory surface area would lead to increased costs for compensatory ion pumping). In addition, challenges such as exercise or hypoxia, causing an increased gill perfusion pressure, could distend already perfused lamellae, thereby decreasing the number of blood cells in near contact with the respiratory water. It has been shown that the thickness of the lamellae increases with pressure (10) and that pillar cells, which keep the "roof" and "floor" of the lamellae together, contain myosin-like microfilaments (3). More recently, actin fibers and actin-binding proteins have been found in eel pillar cells (21). Taken together, these findings strongly suggest that the pillar cell has contractile capacity.

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A small increase in perfusion pressure may decrease diffusion distance in the lamellae (10). However, contraction of pillar cells may be a mechanism that prevents excessive "lamellar ballooning," and it would provide an opportunity to match the functional respiratory area to momentary oxygen needs, thereby reducing osmoregulatory costs. Indeed, utilizing an epi-illumination microscopy technique, Sundin and Nilsson (31) observed that intra-arterial injection of ET-1 resulted in increased pillar cell diameter (indicating contraction) and redistribution of blood flow from the pillar cell region to the marginal channels of the lamellae in rainbow trout. Simultaneously with the branchial events, they observed a large increase in ventral aortic blood pressure (P_{VA}). They suggested that fish have the ability to redistribute lamellar blood flow by local contraction of pillar cells. In later experiments on Atlantic cod (*Gadus morhua*) in which the same technique was used, the ET-1-induced increase in pillar cell diameter occurred in conjunction with a sixfold increase in R_{gill} (29). This effect was completely blocked by the unspecific ET receptor antagonist bosentan.

However, the nature of the ET receptors in the gill vasculature is not clear, particularly with regard to which receptor type is associated with pillar cell contraction. Consequently, the aim of the present study was to clarify the nature of the ET mechanisms in operation in cod gills, with the use of specific agonists, antagonists, and antibodies against ET receptors, in combination with in vivo microscopy, cardiovascular measurements, and immunohistochemistry. In addition, to gain a more generalized picture of ET effects in fish gills, we performed comparative cardiovascular experiments with ET on two additional teleost species, the active mackerel (*Scomber scombrus*) and the more sluggish sculpin (*Myoxocephalus scorpius*).

MATERIALS AND METHODS

Cardiovascular and Branchial Studies

Animals. The experiments were carried out between May and July 2003 at Klubban Marine Biological Station (Uppsala University) in Fiskebäckskil, at the Gullmarsfjord, on the west coast of Sweden. Atlantic cod (300–500 g body wt) were obtained from a local fisherman and kept in tanks (5 cod/m^3). Sculpin (100–150 g body wt) were caught in baited fish traps, and mackerel (200–400 g body wt) were caught by trolling in the Gullmarsfjord. The fishes were kept in a circular (2.5-m-ID) tank that was continuously supplied with fresh seawater (10–14°C). Cod and sculpin were allowed to acclimatize for ≥1 wk before any experiments were conducted; mackerel were used within 4 days of capture.

Surgery. The fish were anesthetized by addition of ethyl aminobenzoate (Benzocaine, NMD, Oslo, Norway), dissolved in 96% ethanol (50 g/l), to the water, to a final concentration of 25 mg/l. Ethyl aminobenzoate has little effect on cardiovascular parameters in fish (11).

After being weighed, the fish was transferred to a surgical table and ventilated with 10°C water containing ethyl aminobenzoate (20 mg/l) through a tube inserted into the mouth.

P_{VA}, P_{DA}, and cardiac output ($\dot{Q}$) were measured as previously described (29, 30). Briefly, a polyethylene (PE) cannula (PE-50 tubing) was inserted into the upper half of the third gill arch between the filaments, into the afferent branchial artery, and pushed ~30 mm toward the ventral aorta. To measure P_{DA}, a 20-mm-long piece of PE-10 tubing, connected to a PE-50 cannula, was inserted into the same gill arch but pushed ~10 mm upward inside the efferent branchial artery toward the dorsal aorta. For measurement of $\dot{Q}$, a single-crystal Doppler flow probe (20 MHz, 45°; Iowa Doppler Products) was placed around the ventral aorta. The Doppler flow probe was connected to a directional pulsed Doppler flowmeter (Department of Bioengineering, University of Iowa).

In mackerel and sculpin, as in cod, the ventral aortic cannula was inserted into the third gill arch. However, in sculpin, the PE-50 cannula was tapered with a section of PE-10 tubing. For measurement of $\dot{Q}$ in sculpin, a small incision was made at the base of the fourth gill arch, and the ventral aorta was exposed to fit a flow probe.

In all three species, the outer half of the right operculum was removed to allow microscopic observations of the gill filaments.

After surgery, the fish was transferred to a Plexiglas container ventilated with water containing ethyl aminobenzoate to maintain anesthesia: 15 mg/l was found optimal for cod and 20 mg/l for sculpin and mackerel. During the first hour after surgery, the hemodynamic variables were allowed to stabilize before any experiments were conducted. The signals from the P_{VA} and P_{DA} preamplifiers and the Doppler flowmeter were recorded on a Powerlab 4/20 (AD Instruments) connected to a laptop computer running Chart version 4.2 (AD Instruments). The same software was used to derive heart rate (HR) from the pulsatile P_{VA}.

To observe microcirculatory changes in the gills, a digital video camcorder (model DCR-PC100E; Sony) was connected to an epi-illumination microscope (Ortholux; Leitz) fitted with a water immersion objective (×22 or ×11; Ultropak, Leitz) as described elsewhere (23, 32). Using this setup, we could observe the circulation in the distal parts of the gill filaments and their lamellae. The diameters of afferent and efferent filament arteries and pillar cells were measured on a video monitor with the use of a slide caliper. Because the images from some individuals were not clear enough to allow quantitative measurements of pillar cell diameter, the cross-sectional areas of several pillar cells were measured in fish where optimal images where obtained.

Table 1. *Primary and secondary antibodies*

Antigen	Antiserum Code	Dilution	Source	Host	Antigen Peptide Sequence	Amino Acid Residues for Antigen
			Primary antisera			
ET	AB-3280	1:100	Chemicon	Rabbit		
ET_A receptor	AER-001	1:100	Alomone Labs	Rabbit	NHNTERSSHKDSMN	413–426
ET_B receptor	AER-002	1:100	Alomone Labs	Rabbit	CEMLRKKSGMQIALND	298–314
Acetylated tubulin	6-11B-1	1:500	Sigma Immunochemicals	Mouse		
			Secondary antisera			
Rabbit IgG	DaR-FITC	1:100	Jackson	Donkey		
Rabbit IgG	DaR-CY3	1:800	Jackson	Donkey		
Mouse IgG	GaM-FITC	1:10	Amersham Life Science	Sheep		

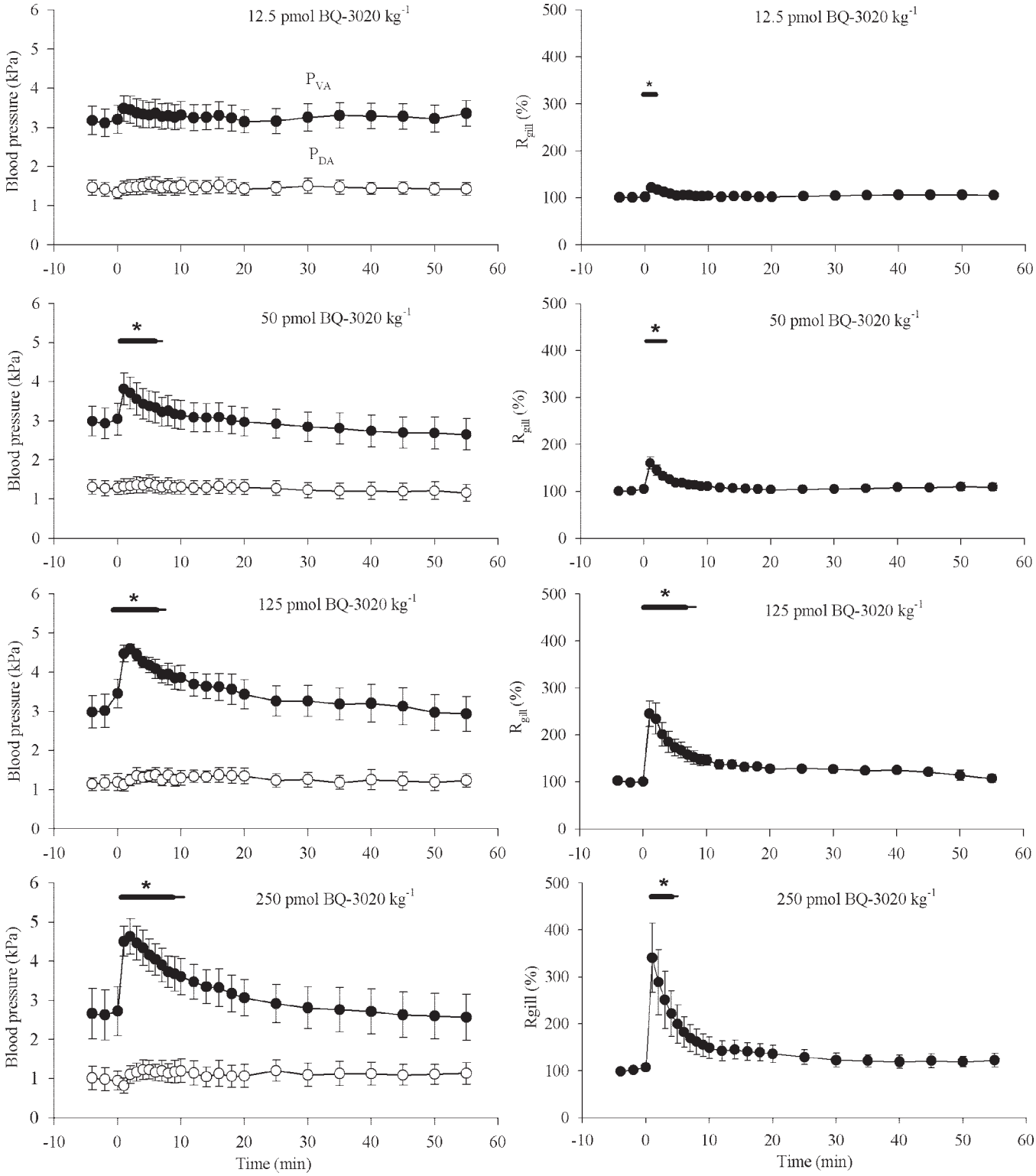


Fig. 1. Dose-dependent cardiovascular responses to endothelin (ET) type B (ET_B) receptor agonist BQ-3020 in cod. *Left column*: ventral and dorsal aortic blood pressure (P_{VA} and P_{DA}) responses to injections of BQ-3020 (12.5, 50, 125, and 250 pmol/kg) into the ventral aorta (at *time 0*). *Right column*: corresponding gill vascular resistance (R_{gill}). Values are means ± SE; $n = 9$, except for 250 pmol/kg ($n = 6$). R_{gill} is given as a percentage, with initial value set to 100. Statistical significance for blood pressure values was tested using repeated-measures ANOVA with Dunnett's posttest. For percent values, a nonparametric repeated-measures ANOVA (Freidman's test) and Dunn's posttest were used. *Significant difference ($P \leq 0.05$) by ANOVA. Horizontal bar, significant posttest period: $P < 0.01$ (thick bar); $P < 0.05$ (thin bar).

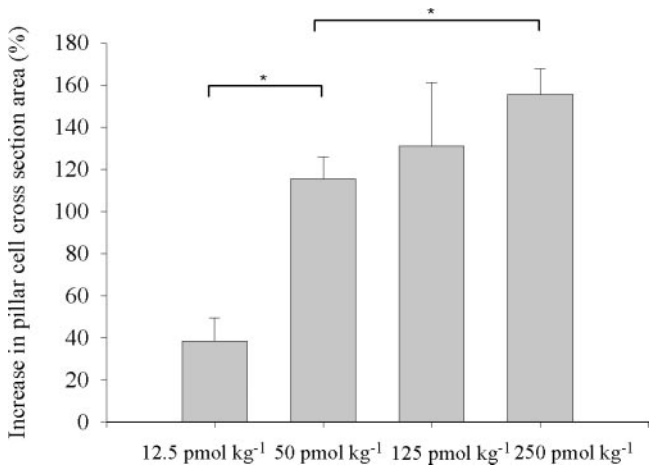


Fig. 2. Dose-dependent increase in pillar cell cross-sectional area with increasing doses of ET_B receptor-specific agonist BQ-3020 injected into ventral aorta of cod. Numbers of cells (*n*) and fish (*N*) are as follows: 12.5 ($n = 6$, $N = 6$), 50 ($n = 8$, $N = 5$), 125 ($n = 9$, $N = 4$), and 250 ($n = 5$, $N = 4$) pmol/kg. Values are means ± SE. *Statistical difference between groups by unpaired Mann-Whitney *U*-test.

Experimental Protocol

Series 1. In *series 1* ($n = 9$), the cardiovascular effects of the ET_B receptor agonist BQ-3020 (Sigma) were examined by serial injections of 12.5, 50, 125, and 250 pmol/kg (1 h between doses) in cod.

Series 2. In *series 2* ($n = 6$), ET-1 (10 pmol/kg; Sigma) was injected into cod before and after the specific ET_A receptor antagonist BQ-610 (100 nmol/kg; Sigma). To our knowledge, no commercial ET_A agonist is available; hence, ET-1 was injected before and after the ET_A antagonist for comparison.

Series 3. In *series 3*, ET-1 effects were examined in sculpin ($n = 3$) and mackerel ($n = 3$) by injection of increasing doses (10, 40, 100, and 200 pmol/kg in sculpin and 40 and 100 pmol/kg in mackerel) into the afferent branchial artery.

All drugs were dissolved in 0.9% saline and mixed to final concentration before injection. The drugs were injected as a bolus injection in three concentrations (10, 100, and 500 ng/ml) to minimize volume effects of the different doses. All drug injections included a subsequent 200-μl injection of saline to flush the cannula. Control injections of 400 μl of 0.9% saline resulted in a small, but significant, increase in $\dot{Q}$ (15.5 ± 2.7%), P_{VA} (from 3.07 ± 0.25 to 3.48 ± 0.27 kPa), and P_{DA} (from 1.57 ± 0.14 to 1.76 ± 0.17 kPa), reaching a maximum 2 min after injection and returning to preinjection values within 15 min. The mean $\dot{Q}$, P_{VA}, and P_{DA} changes after control injections ($n = 19$) were subtracted from the values recorded after drug injection.

Data Analysis

R_{gill} and systemic vascular resistance (R_{sys}) were calculated as follows: $R_{gill} = (P_{VA} - P_{DA})/\dot{Q}$ and $R_{sys} = P_{DA}/\dot{Q}$. Total vascular resistance (R_{tot}) was calculated as follows: $R_{tot} = P_{VA}/\dot{Q}$. Values are means ± SE. Because only relative values were obtained from the system used for measuring $\dot{Q}$, $\dot{Q}$, R_{gill}, and R_{sys} were normalized to 100% at *time 0* (immediately before drug injection). Pillar cell cross-sectional area was estimated by assuming that the cells were circular in shape, and the mean diameter of two measurements (separated by 90°) was used to calculate the area (29).

For detection of statistically significant differences between means of pre- and postinjection values, a repeated-measures ANOVA with a Dunnett's posttest was used for blood pressures and HR. For percent values, a nonparametric repeated-measures (Freidman's) ANOVA with Dunn's posttest was used. The level of significance required to perform a posttest was set to ≤0.05 in all experiments.

Immunohistochemistry

Atlantic cod were captured outside Drøbak in the Oslofjord, Norway, and kept in holding tanks supplied with seawater for several weeks before any experiments were conducted. Four individuals with a body mass of 200–400 g were anesthetized in seawater containing ethyl aminobenzoate (30 mg/l) for 10 min. The heart was rapidly dissected free, and a PE-240 cannula was inserted into the ventricle and pushed forward into the aorta. The fish were perfused with heparinized saline (0.9% NaCl) for removal of erythrocytes from the gills. After ~5 min, the perfusate was changed to a 100-mM phosphate buffer (pH 7.8) containing 4% formaldehyde. After 10 min, the gills where dissected free, placed in PBS containing 4% formaldehyde, and kept at 4°C for 4 h. The fixed gill tissue was then rinsed and stored in PBS containing 30% sucrose until it was used for immunohistochemical staining.

The gill sections were placed in Tissue-Tek (OCT compound; Sekura Finetek Europe, Zoetenwoude, The Netherlands) and quick-frozen in isopentane cooled in liquid nitrogen. Sections (12 μm) were cut using a cryostat (model HM 560; Microm International, Waldorf, Germany) and dried onto gelatin-coated slides. Sections were cut into *1*) longitudinal sections (longitudinal to the gill arch and longitudinal to the filament), *2*) longitudinal cross sections (across the gill arch and longitudinal to the filament), and *3*) cross sections (longitudinal to the gill arch and across the filament). The sections were incubated overnight in a moist chamber at room temperature with the primary antibody listed in Table 1 for single or double staining. Secondary

Table 2. *Ventral aortic blood pressures and pillar cell increase in four species of teleost*

Species	*n*	Blood Pressure			%Pillar Cell Increase
		Control, kPa	Max, kPa	%Increase	
Cod (*Gadus morhua*)					
10 pmol/kg ET-1	6	3.36±0.21	3.87±0.22	15.18	44.18±9.16
40 pmol/kg ET-1*	6	3.60±0.23	4.76±0.27	32.22	93.67±18.09
Rainbow trout (*Oncorhynchus mykiss*)					
80 pmol/kg ET-1†		6.90±0.60	9.20±0.90	33.33	
Sculpin (*Myoxocephalus scorpius*)					
40 pmol/kg ET-1	3	2.15±0.29	2.24±0.16	4.19	
100 pmol/kg ET-1	3	1.95±0.24	2.33±0.19	19.49	51.70±23.93
200 pmol/kg ET-1	3	2.06±0.16	2.66±0.08	29.13	81.74±19.15
Mackerel (*Scomber scombrus*)					
40 pmol/kg ET-1	3	5.96±0.54	6.86±0.77	15.10	
100 pmol/kg ET-1	3	6.53±0.28	7.83±0.54	19.91	

Values are means ± S.E. ET-1, endothelin 1. *Values calculated from Stensløkken et al. (29). †Values calculated from Sundin and Nilsson (31).

antibodies were conjugated to FITC or cyamine dyes (CY3; Jackson, West Grove, PA).

The sections were observed through a fluorescence microscope (model BX60; Olympus) and photographed with a digital camera (model DMX 1200; Nikon).

For control of specificity, sections were preincubated with normal donkey serum (1:10 dilution), incubated without the primary antibody, or preabsorbed with respective antigen. The preadsorption was done according to the manufacturer's protocol (0.75 μg peptide per 1 μg antibody for ET_A and 1 μg peptide per 1 μg antibody for ET_B and ET). No nonspecific binding was observed.

RESULTS

Series 1

Injection of the ET_B receptor agonist BQ-3020 into the ventral aorta of cod produced a dose-dependent increase in P_{VA} (Fig. 1) but no significant changes in P_{DA} or $\dot{Q}$ (data not shown). An increase in P_{VA} and no change in $\dot{Q}$ after BQ-3020 injection resulted in a dose-dependent increase in R_{gill} (Fig. 1). BQ-3020 (50 pmol/kg) increased P_{VA} from 3.04 ± 0.40 to 3.81 ± 0.40 kPa ($P < 0.05$) and increased R_{gill} by 60.1 ± 12.8% ($P < 0.05$). In response to the 125 and 250 pmol/kg injections, P_{VA} increased from 3.45 ± 0.37 to 4.59 ± 0.12 kPa and from 2.72 ± 0.62 to 4.63 ± 0.45 kPa, respectively ($P < 0.05$). The same doses increased R_{gill} by 145.1 ± 67.7% and 240.5 ± 73.9%, respectively. In parallel with the increase in R_{gill}, we observed a redistribution of erythrocytes from the intralamellar space toward the marginal channel, which occurred along with an increase in pillar cell area (Fig. 2). The highest dose of BQ-3020 (250 pmol/kg) resulted in the largest increase in pillar cell area of 155.3 ± 30.5%. The cod displayed some individual differences in sensitivity to the ET_B receptor agonist: a circulatory collapse and cardiac arrest occurred in three of the nine individuals given the highest dose (these fish were excluded from the data set).

Series 2

To ensure recovery after injection, a submaximal dose of ET-1 (10 pmol/kg) was chosen for pre- and post-ET_A receptor antagonist injection. Injection of 10 pmol/kg ET-1 significantly increased P_{VA} by 15.2% (Table 2; $P < 0.05$) and R_{gill} by 40.66 ± 10.48% ($P < 0.05$; Fig. 3). These changes were not significantly affected by pretreatment with the ET_A receptor antagonist BQ-610 (100 nmol/kg). Moreover, the ET_A receptor antagonist was also unable to block the ET-1-induced increase in pillar cell cross-sectional area: 44.18 ± 9.16% (Table 2; $P < 0.05$) and 62.31 ± 15.93% (data not shown) increase without and with antagonist, respectively. In a pilot experiment in which ET-1 (10 pmol/kg) was injected before and after BQ-610 (1 μmol/kg), there was no effect of the ET_A receptor antagonist on the cardiovascular variables.

Series 3

The anesthetized mackerel invariably displayed an intermittent heartbeat pattern: 90–110 beats/min for 30 s, with a 5-s pause in a very regular fashion throughout the experiment (independent of treatment), which lasted for 5 h. Nevertheless, the mackerel were less sensitive than cod (29) to ET-1, and 100 pmol/kg ET-1 produced only a relatively small increase in P_{VA} (Table 2). This dose was tested on only three individuals. Because of the high density of lamellae in mackerel, microscopic observation of single lamellae was difficult, and we found it impossible to obtain quantitative measurements of pillar cell cross-sectional area.

The greater distance between adjacent lamellae in sculpin than in mackerel resulted in much better microscopic images of

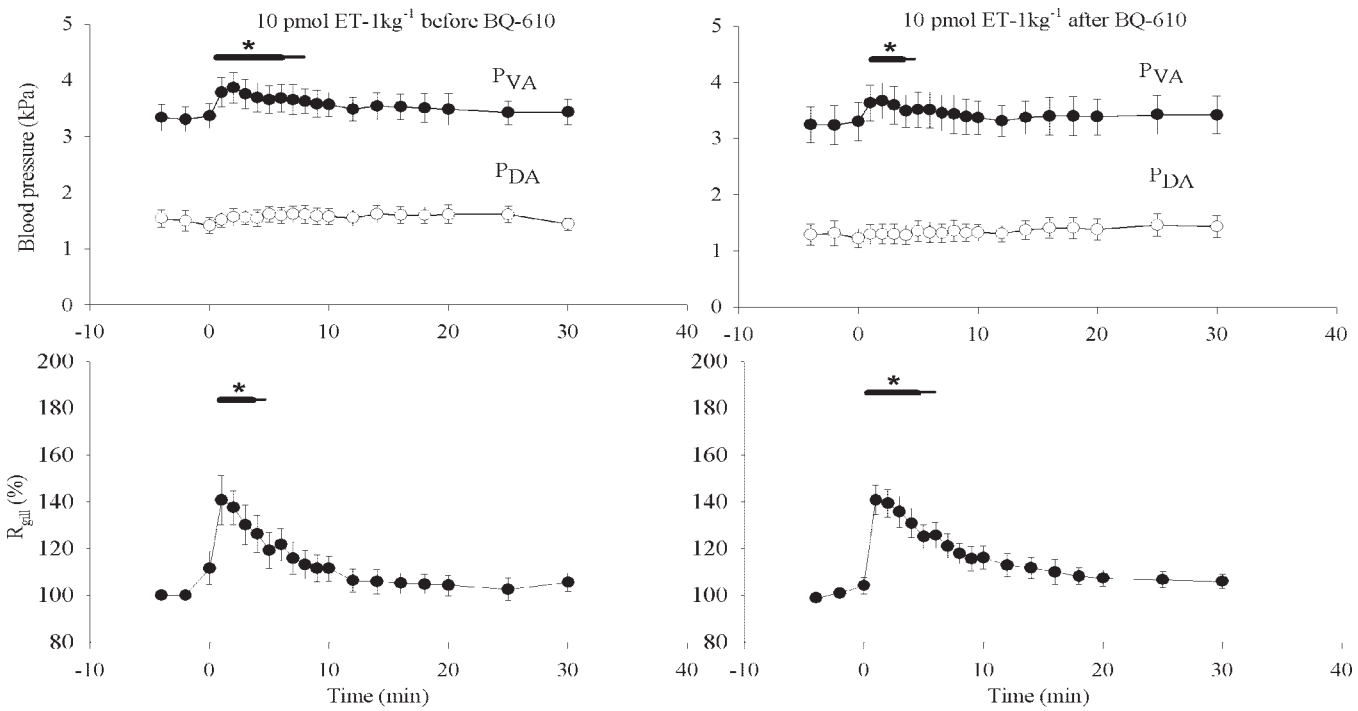


Fig. 3. Blood pressure (P_{DA} and P_{VA}) and R_{gill} changes in response to intra-arterial injection of 10 pmol/kg ET-1 before (*left*) and after (*right*) injection of the ET_A receptor antagonist BQ-610 (100 nmol/kg) in cod. Values are means ± SE; $n = 6$. See Fig. 1 legend for statistical details.

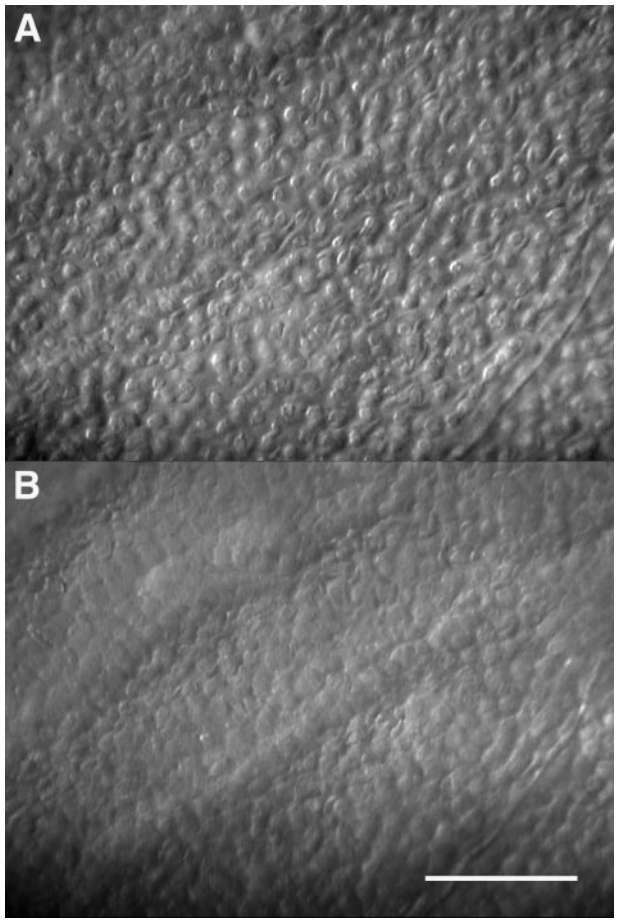


Fig. 4. Micrographs showing a single lamella in sculpin before (*A*) and 60 s after (*B*) intra-arterial injection of ET-1 (200 pmol/kg). Pillar cells are the circular cells that dominate the micrographs. As pillar cell cross-sectional area increases (indicating cell contraction), space between adjacent pillar cells is reduced. Scale bar, 100 μm. (See supplemental videoclip at http://ajpregu.physiology.org/cgi/content/full/00618.2004/DC1.)

the sculpin lamellae (Fig. 4). Also, the sensitivity to ET-1 injections was lower in sculpin than in cod, and 100 pmol/kg ET-1 resulted in only a small increase in P_{VA} (Table 2). Because we placed only a ventral aortic cannula in the sculpin, only R_{tot} was calculated. ET-1 (100 pmol/kg) increased R_{tot} by 39.7 ± 20.1% (data not shown). Simultaneously, a redistribution of blood flow was observed in the lamella. Similar to the responses in cod, ET-1 increased the speed of erythrocytes in the marginal channel in conjunction with an increase in the cross-sectional area of the pillar cells. ET-1 at 100 and 200 pmol/kg increased the diameter by 51.7 ± 23.9% and 81.7 ± 19.1%, respectively (Fig. 4, Table 2).

Immunohistochemistry

ET_B receptor-like IR was found in the lamellae, on one of the sides of the basal membrane connecting to the pillar cells, on the pillar cells flanges or on the basolateral side of the epithelial cell (Fig. 5, *A–E*). Such IR was also seen in NEC (Fig. 5, *A, B*, and *D*), lamellar arterioles (Fig. 5*D*), efferent filament artery (not shown), and afferent filament artery (Fig. 5*F*).

By contrast, very little ET_A receptor-like IR was detected in the lamellar region, although very faint ET_A receptor-like IR adjacent to pillar cells was observed in some lamellae (not shown). Double staining with acetylated tubulin (green) demonstrated that the ET_A receptor antibody (yellowish) used attached to a major part of the branchial nerve fibers IR to acetylated tubulin (Fig. 5, *G–L*). Clear ET_A receptor-like IR was located in nerves fibers running from the base of the filament to the tip (Fig. 5*G*), around lamellar arterioles (Fig. 5*H*), around filament arteries (Fig. 5, *J–L*), and in the central venous system (Fig. 5*I*).

Clear ET-like IR was detected in NEC in the filament tip (Fig. 5*O*) and on or between lamellae (Fig. 5, *M* and *N*).

DISCUSSION

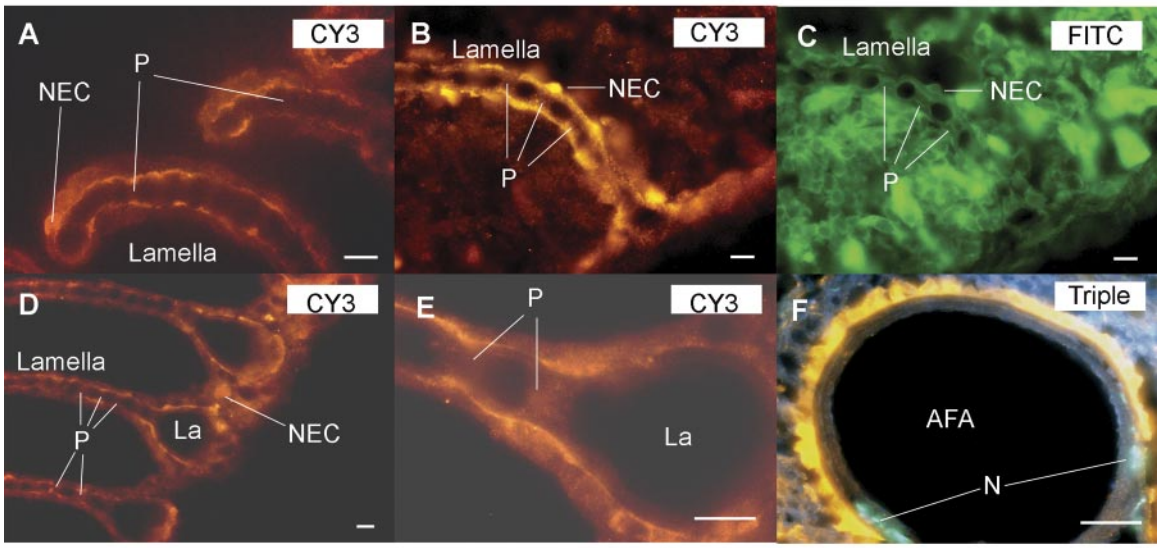
The results show that the specific ET_B receptor agonist BQ-3020 mimics the contractile effect of ET-1 on cod pillar cells. Both peptides induced an increased pillar cell diameter and increased R_{gill}. Together with the finding that the ET_A receptor antagonist BQ-610 was unable to block the ET-1 response, it is likely that an ET_B-like receptor is mediating the pillar cell contraction. In support of this conclusion is the finding that an ET_B-type receptor is probably mediating a contraction of aortic vascular smooth muscle in the spiny dogfish (5). Evans and Gunderson (6) also found evidence for a single ET_B receptor type in the gill of the spiny dogfish. Even though ET-1 may constrict gill vessels (25, 35), the dose-dependent increase in R_{gill} caused by ET-1 is probably, in large part, caused by a contraction of pillar cells, as indicated in previous studies (29, 31). Initial microscopic observation of the filament arteries and arterioles in this study did not reveal changes in the diameter of these vessels after ET-1 or BQ-3020 injection.

The cardiovascular variables differ from those normally reported in instrumented but conscious cod. Thus blood pressures in this study are lower, and although the gill and systemic resistance are similar in our cod, they differ markedly in conscious cod (1, 13, 33). The sympathetic nervous system is excitatory in the vasculature and heart of conscious fish (12, 24, 34). Hence, the low blood pressures can be a result of the anesthesia inhibiting the sympathetic nervous system. However, because we report changes compared with preinjection values and because the changes are large, our findings are still physiologically relevant.

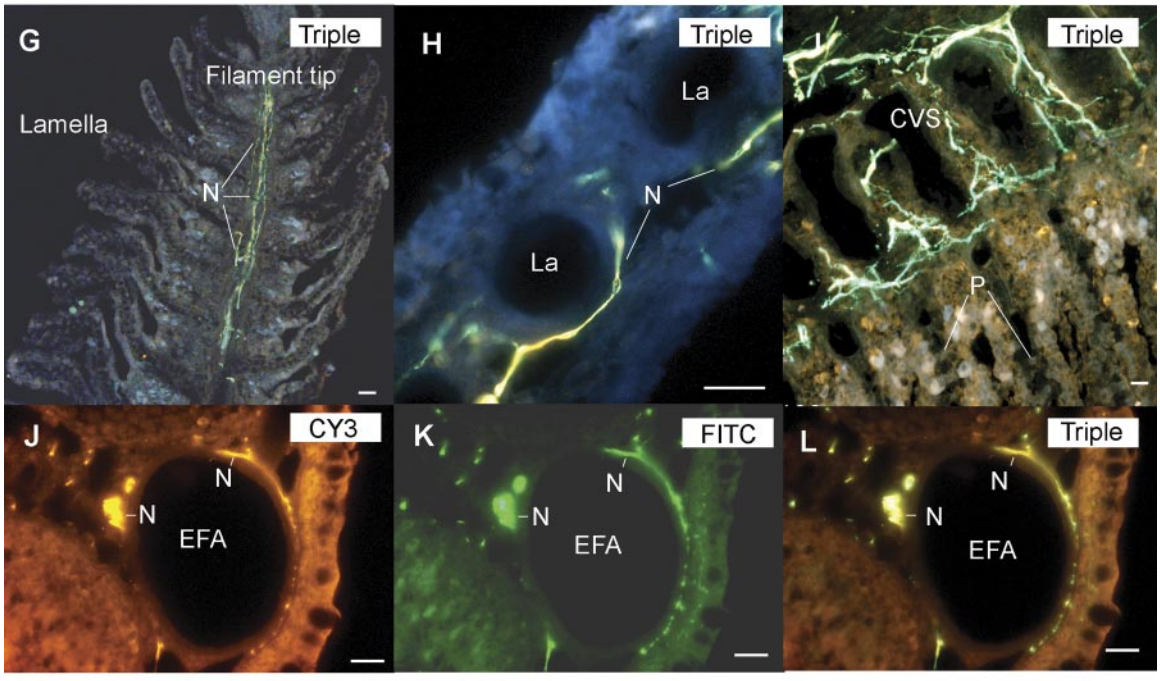
Unfortunately, there is no commercially available ET_A receptor agonist. Instead, we used ET-1 as an agonist and BQ-610 as a ET_A receptor blocker, at a dose that blocks ET_A receptors in mammals (2, 16), to investigate involvement of the ET_A receptor in pillar cell contraction. Our finding that BQ-610 did not reduce the ET-1-induced pillar cell contraction or increase R_{gill} also indicates that an ET_B receptor is involved in this response. However, we cannot rule out the possibility that the cod ET_A receptor could structurally differ from the mammalian receptors and that the mammalian ET_A antagonist was unable to block the cod ET_A receptor. Molecular characterization of fish ET receptors is clearly needed.

Although the same cardiovascular response pattern was observed, a higher dose of ET-1 was required to elicit a response in mackerel and sculpin. A reason for the lesser sensitivity in the sculpin could be that the distance between individual pillar cells is greater than that in the cod, so that even when they are maximally contracted, blood would still flow between the pillar cells. Indeed, despite a marked pillar

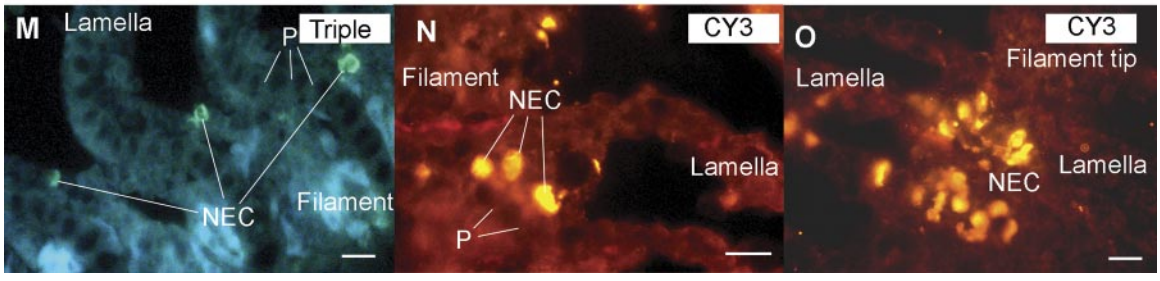
ET_B-receptor immunoreactivity



ET_A-receptor immunoreactivity



ET immunoreactivity



cell contraction, only a small but apparent increase in P_{VA} and R_{tot} was seen after ET-1 injection in the sculpin. However, the much more active mackerel, with its close spacing of pillar cells, also displayed a low sensitivity to ET-1. Unfortunately, the high density of lamellae in mackerel obscured the microscopic view of the individual lamellar sheets, so it was impossible to assess the magnitude of pillar cell contraction in this species. However, species differences in, for example, receptor density and contractility of pillar cells are likely. Nevertheless, contraction in response to ET-1 appears to be a general feature of fish pillar cells. With available results, which are limited to the sluggish sculpin, cod (this study and Ref. 25), rainbow trout (31), and the very active mackerel, we cannot correlate the magnitude of ET-induced pillar cell contractions with factors such as lifestyle or habitat choice.

It has been suggested that, in fish at rest, roughly two-thirds of the lamellae are normally perfused (4, 9). However, more lamellae may be recruited if mean and/or pulse pressure is increased, as suggested by Farrell et al. (10). It is possible that our observed contraction of pillar cells is a mechanism aimed at preventing lamellar ballooning in already perfused lamellae, thereby reducing diffusion distance between the erythrocytes and the water and, thus, facilitating gas exchange. On the other hand, contraction of pillar cells may also decrease the respiratory surface area by reducing lamellar perfusion during periods of low activity and/or high oxygen availability, thereby avoiding energetically costly ion fluxes, as suggested by Sundin and Nilsson (31). Of course, these possible roles of pillar cell contraction are not necessarily mutually exclusive.

The cardiovascular experiments were corroborated by immunohistochemical data showing abundant ET_B receptor-like IR in conjunction with the basal lamina adjacent to the pillar cells (Fig. 5, *A–E*). Unfortunately, it was difficult to conclude whether the IR was on the outer margin of the pillar cell flanges or on the basolateral side of the epithelial cells (Fig. 5*E*). It is surprising that the receptors are not detected on the luminal side of the lamellae adjacent to the blood and the putative released ET. Still, because it is a small peptide, it is possible that ET-1 in blood can reach the receptors between the pillar cell flanges by way of diffusion or even through transport mechanisms. Nevertheless, the mechanisms involved in the pillar cell contraction are not known, but our finding adds some information. Moreover, ET-1 may work in a paracrine/autocrine fashion (see below).

Occasionally, some weak IR of the ET_A receptor antibody was also observed on or near pillar cells. We can probably rule out cross-reactivity, because the epitopes for these two antibodies do not share more than one amino acid residue. The ET_B receptor sequence in zebrafish (*Danio rerio*) (26, 27) shows that, for the amino acid sequence of the epitope toward which the antibody was produced, 14 of 17 are identical in zebrafish and rat. For the ET_A receptor, the specificity of the antibody we have used is difficult to evaluate, because no known teleost DNA sequence closely resembles that of the rat ET_A receptor (according to the National Center for Biotechnology Information database). On the other hand, there appear to be no other known sequences of other proteins that the ET_A receptor antibody should be able to recognize (as indicated by a search in the National Center for Biotechnology Information database).

In contrast to the location of the ET_B receptors in conjunction with the basal lamina, ET labeling was only found in NEC situated on the lamellae buried in the filament (Fig. 5*N*) and between each lamella. Typically, one cell was stained between adjacent lamellae, although more NEC were observed toward the filament tip. These cells are probably the same as those described by Zaccone et al. (40), who first demonstrated ET storage in NEC in fish gills. In addition, we have shown that some of these NEC also exhibit ET_B-like receptors (Fig. 5, *A, B*, and *D*), implying an autocrine regulation of these cells. Given that NECs on the lamellae are in close contact with the blood and contain ET (Fig. 5*N*), it is possible that factors such as oxygen status, cause NECs to release ET, thereby affecting the size of the functional respiratory surface area by contracting the pillar cells.

An interesting immunohistochemical finding was the strong ET_A receptor IR in nerve fibers (Fig. 5, *G–L*), indicating a role for ET, other than vasoregulation, in fish gills. To our knowledge, this is the first evidence of ET_A receptors in fish nerves. To verify that the ET_A receptor IR was specific for nerves, the gill sections were double stained for acetylated tubulin, which has been found to be specific for nerve cells in cod (28). We found ET_A receptor IR in nerve fibers running along filament arteries and lamellar arterioles and forming a network around the central venous system of the filaments, and it is possible that ET is involved in the transmission of sensory signals via ET_A receptors, as found in mammals (14, 41). ET receptors are also found in cervical and nodose gangalia and in the carotid body in mammals, implying a role in cardiovascular baro- and chemoreflexes (22). There is also evidence that postganglionic sympathetic neurons express functional ET receptors that may modulate the release of neurotransmitter from the nerve terminals (38). Despite the recent evidence of an extensive lamellar innervation in zebrafish, probably innervating NEC (17), we found no ET_A receptor-like IR on nerve fibers in the lamellae.

Fig. 5. Images showing immunoreactivity (IR) of antibodies against ET_B and ET_A receptors, ET, and acetylated α-tubulin-labeled nerves in Atlantic cod gills. Images appear orange when the secondary CY3 antibody was employed for visualization through the CY3 filter (excitation = 545 nm, dichromatic lamina = 565 nm, barrier = 610 nm); they appear green when the secondary FITC antibody was employed for visualization through the FITC filter. Where FITC and CY3 are visualized with a triple filter (excitation = 420 nm, dichromatic lamina = 415 nm, barrier = 465 nm), images are blue/orange. Filters are indicated at *top right* in each image. Scale bar, 10 μm. AFA, afferent filament artery; EFA, efferent filament artery; CVS, central venous system; FA, filament artery; La, lamellar arteriole; NEC, neuroendocrine cell; N, nerves; P, pillar cell. *A–F*: ET_B receptor-like IR. Note yellowish IR in conjunction with pillar cells in lamellae (*A, B, D*, and *E*). *C*: same as *B*, but observed through FITC filter. It shows a more detailed view of pillar cells and also serves as a control for autofluorescence of labeling in *B*. NEC can be seen between adjacent lamellae (*D*) and on lamellae (*A* and *B*). *F*: double labeling viewed through a triple filter with ET_B (orange) receptor-like IR and acetylated α-tubulin-like IR (green) on the AFA. ET_B antibody labels cells on the outer muscle layer but not nerve fibers on the AFA. *A–C* are longitudinal sections, *D* and *E* are longitudinal cross sections, and *F* is a cross section. *G–L*: double labeling with antibodies against acetylated α-tubulin (green) and ET_A receptors (orange). Double-labeled nerve fibers appear yellow/white. ET_A receptor-stained nerve fibers travel to the filament tip (*G*) and surround lamellae and the CVS (*H* and *I*). *J* and *K*: same image visualized with all 3 filters to show extensive double labeling. ET_A receptor-like IR (*J*), acetylated α-tubulin-like IR (*K*), and double labeling (*L*) around the EFA. *G* and *H* are longitudinal sections, *I* is longitudinal cross section, and *J* and *L* are cross sections. *M–O*: ET-like IR on NEC between adjacent lamellae (*M*), in conjunction with the pillar cells (*N*), and in clusters in the filament tip (*O*). *M* is a longitudinal section; *N* and *O* are longitudinal cross sections.

In conclusion, it appears to be a general feature among teleost fishes that pillar cells are sensitive to ET and that their contraction causes a redistribution of lamellar blood flow away from the lamellar sheet toward the marginal channels. This probably allows an adjustment of the functional respiratory surface area. The results clearly indicate that the contraction is mediated by an ET_B-like receptor in cod.

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