ARTERIO-VENOUS BRANCHIAL BLOOD FLOW IN THE ATLANTIC COD *GADUS MORHUA*

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Summary

We have estimated the branchial venous blood flow in the Atlantic cod by direct single-crystal Doppler blood flow measurements *in vivo*. In the undisturbed animal, this flow amounts to $1.7\,\mathrm{ml\,min^{-1}\,kg^{-1}}$, which corresponds to about 8 % of the cardiac output. Studies of both an isolated perfused gill apparatus *in situ* and simultaneous measurements of cardiac output and branchial venous flow *in vivo* were made to assess the effects of some putative vasoregulatory substances. Adrenaline dilates the arterio-arterial pathway and constricts the arterio-venous pathway, thus decreasing branchial venous drainage. 5-Hydroxytryptamine (5-HT), in contrast, produced marked vasoconstriction in the arterio-arterial pathway of the branchial vasculature, increasing the branchial venous blood flow. Cholecystokinin-8 (CCK-8) and caerulein produced similar cardiovascular effects, with marked constriction of both arterio-arterial and arterio-venous pathways. The study demonstrates the ability of the vascular system of the gills to regulate the distribution of branchial blood flow, and summarizes the vasomotor effects of some substances with possible vasomotor function in the cod gills.

Introduction

Blood entering the branchial circulation of the cod through the afferent branchial arteries may leave the gills *via* two different routes after passing the lamellae: (1) *via* the efferent filamental arteries, which run to the suprabranchial arteries distributing blood to the systemic circulation *via* the dorsal aorta (arterio-arterial pathway), or (2) *via* the branchial veins into the inferior jugular veins ventrally and the anterior cardinal veins dorsally (arterio-venous pathway). It is believed that the arterio-arterial pathway is important for respiration and distribution of oxygenated blood to the systemic circulation. The arterio-venous pathway includes the nutritive vascular supply to the gills, and may also be important in ion regulation. In addition, an arterio-venous shunt may be used to increase the flow of oxygenated blood back to the heart (Laurent, 1984; Nilsson, 1984; Randall, 1985). Control of the distribution of the blood between the two

pathways is therefore of paramount importance in balancing the respiratory and ion-transporting functions of the gills (Laurent and Dunel, 1980).

The size of the branchial venous flow has been the subject of some debate (Randall, 1985) and, by using a relative calculation method based on differences in haemoglobin concentration between the two circuits, the arterio-venous blood flow in the rainbow trout (*Oncorhynchus mykiss*) has been estimated to be $7.0\pm4.7\%$ (mean$\pm$s.e.) of cardiac output (Ishimatsu *et al.* 1988).

A model for the autonomic nervous control of the gill vasculature can be derived from histochemical and ultrastructural studies of the distribution of nerves within the gills (Donald, 1984, 1987; Bailly and Dunel-Erb, 1986; Dunel-Erb and Bailly, 1986; Nilsson, 1986; Bailly *et al.* 1989), in conjunction with physiological and pharmacological studies (Payan and Giraud, 1977; Smith, 1977, 1978; Pettersson and Nilsson, 1979; Nilsson and Pettersson, 1981). From these studies, it was established that cholinergic vasoconstrictor fibres innervate the sphincter at the base of the efferent filamental artery, while adrenergic fibres run to the afferent filamental artery and lamellar arterioles as well as to the nutritive vasculature of the gill arch and to the central venous system. The most prominent effect of the adrenergic innervation of the branchial vasculature appears to be an α-adrenoceptor-mediated vasoconstriction of the arterio-venous pathway (Nilsson and Pettersson, 1981), and a major function of circulating (humoral) catecholamines is to dilate the arterio-arterial pathway *via* an action on β-adrenoceptors (Nilsson and Pettersson, 1981; Wahlqvist, 1980, 1981; Nilsson, 1986).

Histochemical studies have revealed a vascular innervation by serotonergic [5-hydroxytryptamine (5-HT)-containing] nerve fibres, notably of the sphincter in the efferent filamental artery. 5-HT is also present in neuroepithelial cells (Dunel-Erb *et al.* 1982; Bailly *et al.* 1989). It is known that 5-HT is a potent vasoconstrictor in fish gills (Östlund and Fänge, 1962; Reite, 1969; Katchen *et al.* 1976).

In addition to the amine neurotransmitters, recent observations suggest the presence of a gastrin/CCK/caerulein-like peptide in vasomotor nerves within the gills of the Atlantic cod, *Gadus morhua* (S. Holmgren, unpublished data).

This study was performed in an attempt to quantify branchial venous blood flow, by measuring directly the inferior jugular vein flow, and to assess the effects of adrenaline, 5-HT, CCK-8 and caerulein on the distribution of the blood flow in the gills, using both *in situ* and *in vivo* techniques.

Materials and methods

Atlantic cod (*Gadus morhua* L.) of either sex, with a body mass of 400–1000 g, were used in the experiments. The fish were kept in well-aerated recirculating sea water at 10–12°C prior to the experiments.

In situ *perfused gill arches*

The vasoactivity of adrenaline, 5-hydroxytryptamine (5-HT), cholecystokinin-8

(CCK-8) and caerulein was investigated using an isolated perfused gill apparatus preparation similar to that employed by Nilsson and Pettersson (1981).

Heparin (0.2–0.3 ml; 5000 i.u. ml^{-1}) was injected into the caudal vessels, and the fish was killed by a sharp blow to the head. The right-side gill arches were perfused by cannulating the ventral aorta for inflow of buffered cod Ringer's solution (Holmgren and Nilsson, 1974), delivered at constant flow from a pneumatically driven peristaltic perfusion pump. The fluid was continuously gassed with oxygen/carbon dioxide (97 % O_2/3 % CO_2). Inflow pressure (P_I) was measured using a Statham P23 pressure transducer connected to the inflow *via* a three-way stopcock. At the start of each experiment, the flow was adjusted to give a P_I similar to the ventral aortic pressure of the cod (3.0–5.0 kPa). Outflow counter pressure was kept constant at 1.0 kPa (arterial) and 0 kPa (venous) (see Nilsson and Pettersson, 1981). Pressure calibration was performed against a static water column. For collection of the arterial perfusate, a cannula was inserted into the suprabranchial artery *via* the coeliac-mesenteric artery (Pettersson and Nilsson, 1979). The venous perfusate was collected *via* a cannula in the right inferior jugular vein, near the duct of Cuvier (Nilsson and Pettersson, 1981).

The arterial and venous outflows of perfusion fluid were determined by photoelectric drop counters connected to a tachograph (Grass 7 P4) on a recorder (Grass Polygraph model 7). The right suprabranchial artery was ligated anterior to the first right efferent branchial artery, all gill arches on the left side were ligated and, finally, the left inferior jugular vein was ligated to direct, as far as possible, the perfusate to the right inferior jugular vein. During the experiment, the fish was kept in a temperature-controlled container at 12 °C.

All drugs were given as bolus doses in 50 µl volumes, injected *via* a three-way stopcock into the inflow perfusion line. The dose–response relationship was plotted as percentage flow decrease or increase in relation to pre-injection values.

In vivo *experiments*

To estimate the inferior jugular vein flow *in vivo* and elucidate the effect of the putative vasomotor transmitter substances, fish were anaesthetized in sea water containing 100 mg l^{-1} tricaine methanesulphonate (MS-222; Sigma) until breathing movements ceased. They were then transferred to an operating table where sea water with 50 mg l^{-1} MS-222 was continuously pumped over the gills during surgery.

To measure cardiac output, a midline incision was made anterior to the common branchial and pharyngo-cutaneous aperture. The anterior part of the heart was exposed and the ventral aorta dissected free from surrounding tissue. A single-crystal Doppler flow probe was placed around the ventral aorta, the incision was sutured and the leads from the flow probe were secured by skin sutures.

Incisions were made at the base of the fourth gill arch on both sides of the fish to expose the inferior jugular veins, and the larger of the two veins was chosen for measurement of inferior jugular vein flow. A silicone Doppler flow probe (i.d. 1.0–1.6 mm) was placed around the vein, and the incision was carefully closed

around the probe with sutures. The leads were secured by skin sutures. The smaller vein was then ligated, and the incision on that side was also closed by sutures. An anastomosis connects the right and left veins and, assuming a very small drainage *via* the narrow dorsal venous connections to the anterior cardinal veins, nearly all the branchial venous flow will pass the probe. The Doppler probes were attached to a Doppler flowmeter (Iowa University) which was connected to a Grass Polygraph recorder model 7.

Finally, a polyurethane cannula (PU 90) tipped with a polyethylene cannula (PE 50) and filled with heparinized (100 i.u. ml^{-1}) 0.9 % NaCl was occlusively inserted into the afferent branchial artery of the third gill arch for injection of drugs and to measure ventral aortic pressure. Heart rate was derived from the pulsatile blood pressure signal and displayed using a Grass tachograph preamplifier.

Drugs were injected in volumes of 1 ml kg^{-1}. Injection of this volume of NaCl solution by itself produced a small increase in inferior jugular vein blood flow ($\dot{q}_{IJV}$) and cardiac output ($\dot{Q}$), but this effect of volume injection was transient and did not affect the recorded effects of the drugs. The time interval between injections was adjusted so that the flows were allowed to reach steady levels before the next injection.

After surgery, the fish was allowed to recover for at least 24 h before any experiments were conducted. During this time the effects of anaesthesia and handling wore off and the cardiovascular parameters reached steady levels (Smith *et al.* 1985).

The Doppler flowmeter used in the work accurately measures blood flow velocity in the flow ranges studied and displays this velocity in kHz Doppler shift. There is a direct relationship between the blood velocity and instantaneous volume flow, and previous studies in this laboratory on the cod visceral arteries and on ventral aortic blood flow in the hagfish (Axelsson *et al.* 1990; Axelsson and Fritsche, 1991), in which the mean blood flow was calibrated in absolute terms, have demonstrated a high degree of linear correlation between the Doppler signal and mean volume flow.

In six of the *in vivo* experiments the Doppler flow probes were calibrated *in situ*. Calibration of the inferior jugular vein was made by inserting an inflow cannula into the vein and an outflow cannula into the duct of Cuvier taking care not to displace the flow probe. The ventral aorta was then similarly prepared by inserting an inflow cannula into the ventricle and an outflow cannula into the ventral aorta anterior to the probe. Diluted, heparinized cod blood was used to calibrate the probes over a range of flows covering the lowest mean flow value to the highest peak value recorded for that animal. The data were then analyzed by linear regression analysis to provide calibration values.

Drugs

The following drugs were used: L-adrenaline bitrartrate (Sigma), 5-hydroxytryptamine creatinine sulphate (Sigma), sulphated cholecystokinin-8

(Research Plus) and sulphated caerulein (Bachem). Peptides were dissolved in stock solutions in 0.9% NaCl, containing 0.002% merthiolate and $1\,mg\,ml^{-1}$ albumin. Solutions for injection were diluted with 0.9% NaCl.

Calculations and statistical analyses

Blood flow, expressed as $ml\,min^{-1}\,kg^{-1}$, was calculated from the recorded Doppler shift (kHz) by linear regression analysis of calibration values from six animals. The regression coefficient for each fish was always greater than 0.95.

Evaluation of statistically significant differences ($P<0.05$) in the observations was made using the Wilcoxon signed-rank test. Asterisks in the diagrams indicate statistically significant differences. The results are presented as means±S.E.M.

Results

In situ *perfused gill arches*

The responses to adrenaline are well documented (e.g. Nilsson and Pettersson, 1981), and the drug (1 nmol) was used to confirm the condition of the preparation throughout the duration of the experiment. As in the previous experiments, adrenaline produced a biphasic effect on P_1 with a marked reduction in the arterio-venous flow while the arterio-arterial flow increased (Fig. 1).

The responses to 5-HT in the *in situ* perfused gill arches were variable. The majority of the preparations showed responses similar to that shown in Fig. 1, with a decrease in both outflows while input pressure increased (see also Fig. 2). In a few preparations, the outflow showed a biphasic change with a transient initial decrease followed by an increase and, in a third group, there were only increased outflows in the dorsal aorta and the vein.

Bolus doses of CCK-8 and caerulein caused a dose-dependent decrease of the jugular vein ($\dot{q}_{JV}$) outflow and also a dramatic reduction in efferent arterial flow ($\dot{q}_{DA}$), concomitant with an increase in inflow counter-pressure (P_1) (Figs 1 and 2).
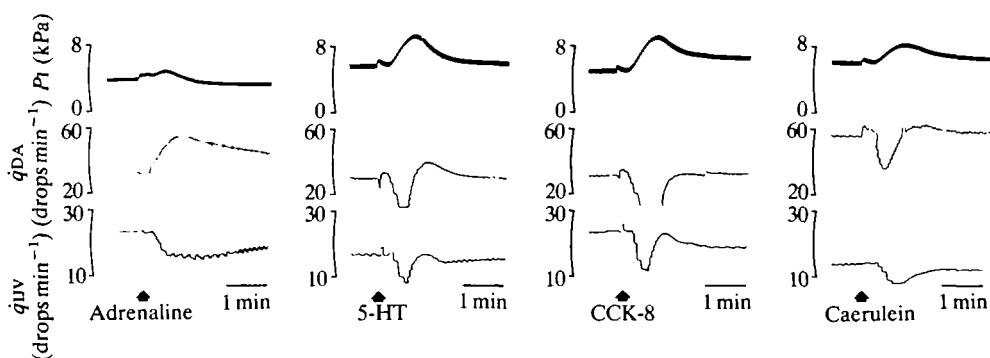

Fig. 1. Perfused right-side gill apparatus *in situ*. Effects of adrenaline (5 nmol), 5-hydroxytryptamine (5-HT, 0.5 nmol), cholecystokinin-8 (CCK-8, 0.5 nmol) and caerulein (0.005 nmol) on the right-side gill apparatus. Inflow pressure P_1 is expressed in kPa and outflow rates ($\dot{q}_{DA}$, outflow to the dorsal aorta; $\dot{q}_{JV}$, outflow to the jugular vein) in drops min^{-1} (10 drops=0.32 ml).
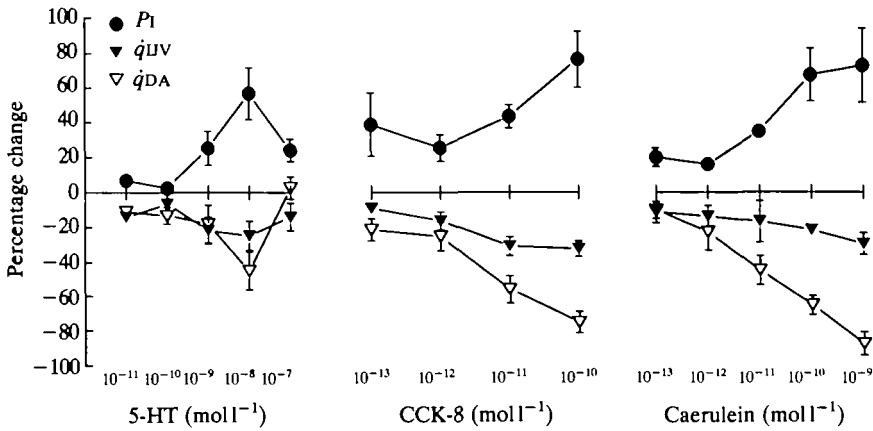
Fig. 2. Perfused right-side gill apparatus *in situ*. Dose–response curves for 5-HT, CCK-8 and caerulein on the outflow in the dorsal aorta ($\dot{q}$DA, drops min^{-1}) and inferior jugular vein ($\dot{q}$IJV, drops min^{-1}) and the ventral aortic input pressure (PI, kPa). The diagram shows responses as a percentage of the pre-injection value. Means±S.E.M.; each point illustrates tests on 1–9 animals.

In vivo *experiments*

The inferior jugular vein flow ($\dot{q}$IJV) was estimated at $1.7\pm1.3\,\text{ml}\,\text{min}^{-1}\,\text{kg}^{-1}$ ($N=6$) in the present experiments and, with a cardiac output ($\dot{Q}$) of $20.4\pm3.2\,\text{ml}$ $\text{min}^{-1}\,\text{kg}^{-1}$ ($N=6$), this corresponds to $8.2\pm1.1\,\%$ of $\dot{Q}$.

In the living fish, the effect of adrenaline ($\geqslant10\,\text{nmol}\,\text{kg}^{-1}$) on the heart was a transient bradycardia (probably due to a reflex response from the elevated blood pressure), followed by an increase in cardiac output and a marked reduction in $\dot{q}$IJV (Figs 3 and 5).

5-HT (0.01–$10\,\text{nmol}\,\text{kg}^{-1}$) produced an increase in $\dot{Q}$, and there was also a large increase in $\dot{q}$IJV (Figs 4 and 5). The response *in vivo* was similar in all fish, and the differences in the effects on the jugular vein outflow seen in the *in situ* perfusions did not occur.

The effects of CCK-8 (0.1–$10\,\text{nmol}\,\text{kg}^{-1}$) and caerulein ($0.01$–$1\,\text{nmol}\,\text{kg}^{-1}$) *in vivo* support the results obtained in the perfusion experiments. There was a marked reduction in $\dot{q}$IJV, and ventral aortic blood pressure (PVA) and heart rate (fH) increased. Cardiac output decreased as a result of reduced stroke volume (Figs 4 and 5).

Discussion

The cardiac output recorded in the present experiments (approximately $20\,\text{ml}\,\text{kg}^{-1}\,\text{min}^{-1}$) is comparable to the results of previous studies (Axelsson, 1988; Axelsson and Nilsson, 1986; Fritsche and Nilsson, 1989, 1990), and the observed resting arterio-venous branchial flow of $1.7\,\text{ml}\,\text{min}^{-1}\,\text{kg}^{-1}$ corresponds to
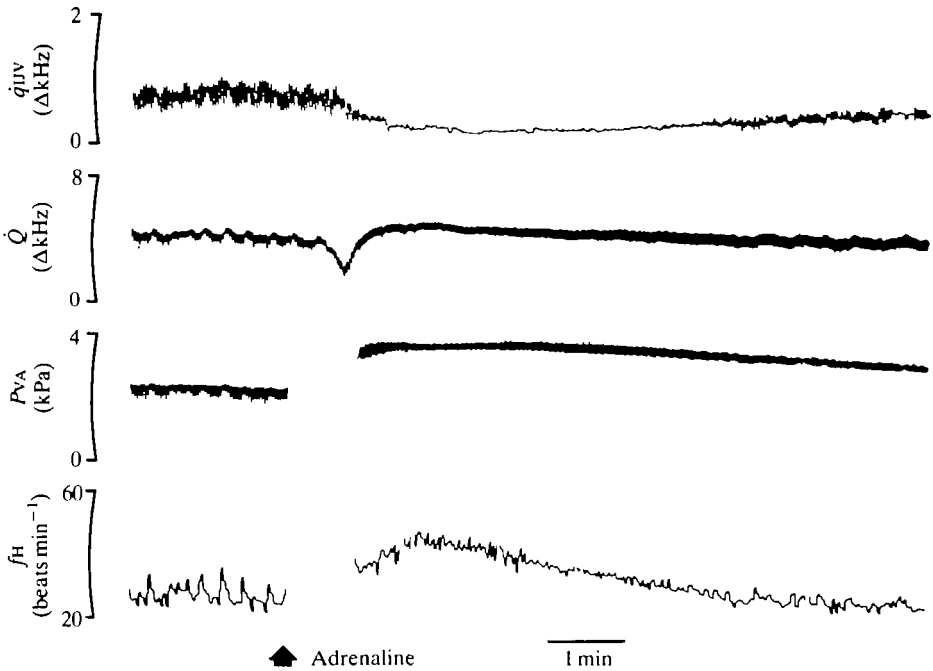
Fig. 3. Unanaesthetized cod *in vivo*. Effects of $100 \, \text{nmol} \, \text{kg}^{-1}$ adrenaline on cardiac output ($\dot{Q}$), inferior jugular vein blood flow ($\dot{q}_{IJV}$), ventral aortic blood pressure (P_{VA}) and heart rate (f_H). Changes are given as ΔkHz Doppler shift.

8.2 ± 1.1 % of cardiac output in the cod. This result, in turn, is quite near the value of 7 % reported for the rainbow trout (Ishimatsu *et al.* 1988).

The responses to adrenaline in the perfusion experiments were similar to those previously described by Nilsson and Petterson (1981), who demonstrated mechanisms involving both α- (predominantly in the arterio-venous pathway) and β-adrenoceptors (predominantly in the arterio-arterial pathway). *In vivo*, effects on f_H due to a barostatic reflex were transient or absent, and there was an increase in $\dot{Q}$. The effect on $\dot{q}_{IJV}$ *in vivo* corroborates the observations from the present perfusion experiments and those of Nilsson and Pettersson (1981), confirming that there is a drastic reduction in branchial venous flow despite the increased cardiac output.

The responses to 5-HT were variable *in situ*. Often a decreased outflow in both the dorsal aorta and the jugular vein occurred, together with an increased inflow pressure. Sometimes, however, biphasic changes or an increased venous flow were observed. The most common response, a constriction of the branchial vasculature, is in agreement with the observations of an increased general branchial resistance observed in several species of teleosts (Östlund and Fänge, 1962; Reite, 1969; Katchen *et al.* 1976).

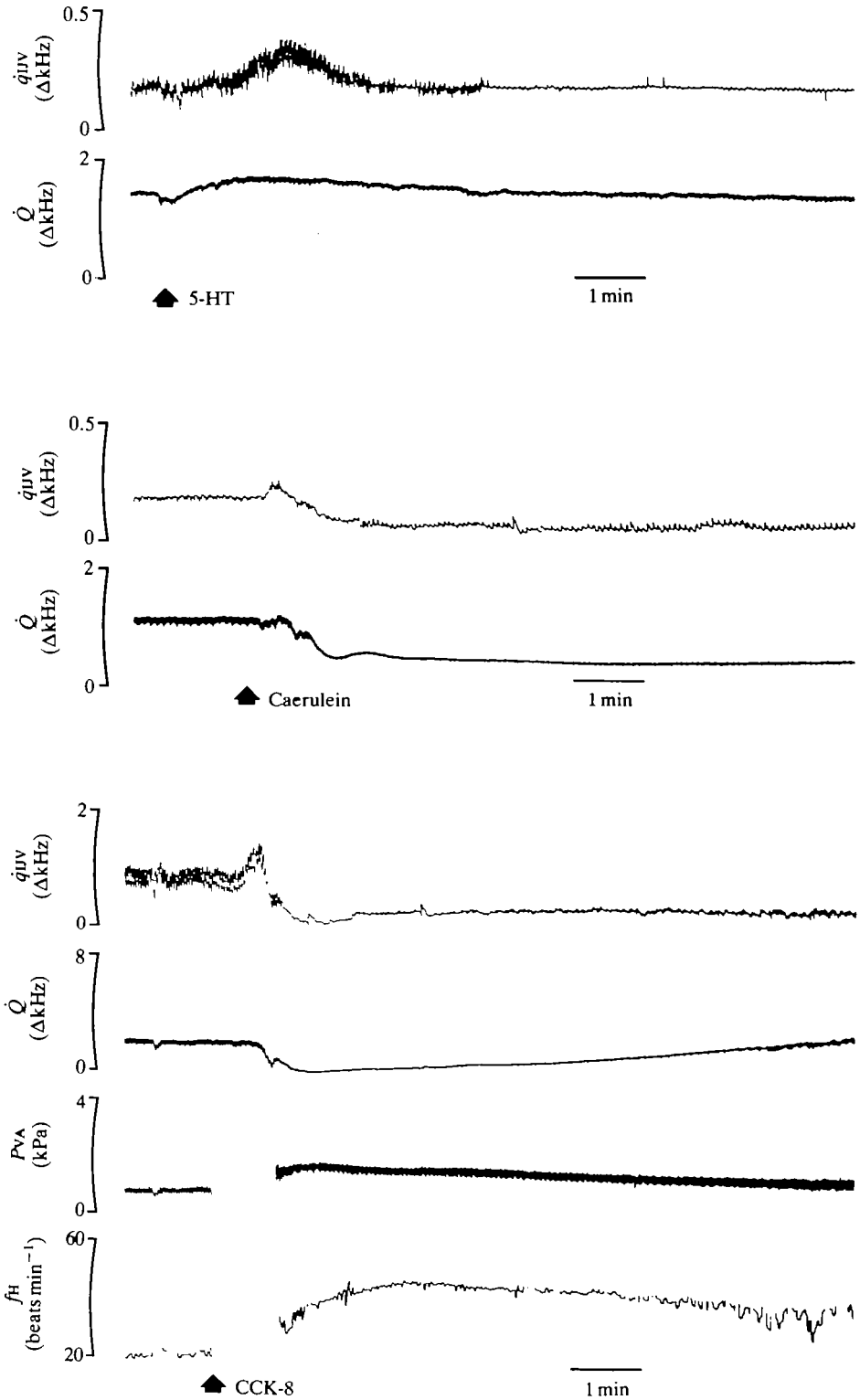*In vivo*, in contrast, 5-HT consistently produced a small dose-dependent

Fig. 4

Fig. 4. Unanaesthetized cod *in vivo*. Effects on cardiac output ($\dot{Q}$), inferior jugular vein blood flow ($\dot{q}_{IJV}$), ventral aortic blood pressure (P_{VA}) and heart rate (f_H) of CCK-8 (10 nmol kg^{-1}), caerulein (1 nmol kg^{-1}) and 5-HT (0.1 nmol kg^{-1}). Changes are given as ΔkHz Doppler shift.
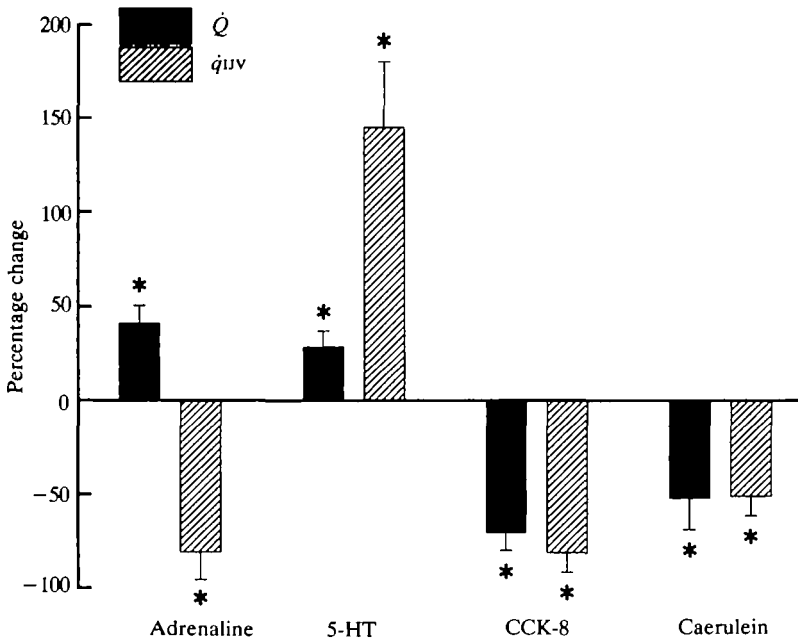

Fig. 5. Unanaesthetized cod *in vivo*. Summary of the effects on cardiac output ($\dot{Q}$) and inferior jugular vein flow ($\dot{q}_{IJV}$) of adrenaline (100 nmol kg^{-1}; $N=7$), 5-HT (0.1 nmol kg^{-1}; $N=7$), CCK-8 (10 nmol kg^{-1}; $N=6$) and caerulein (1 nmol kg^{-1}; $N=6$). The responses are plotted as the maximum response as a percentage of the pre-injection value. Means±s.E.M. Asterisks denote a value significantly different from the pre-injection value ($P<0.05$).

increase in $\dot{Q}$ and a very prominent increase in $\dot{q}_{IJV}$. Serotonergic neurones have been demonstrated in the efferent filamental artery sphincter in teleosts (Bailly *et al.* 1989). One simple explanation of the increased flow through the arterio-venous pathway *in vivo*, is a 5-HT-induced constriction of this sphincter, which will redirect the blood towards the arterio-venous pathway.

The reason for the discrepancy in the effects of 5-HT between *in situ* and *in vivo* experiments is not clear. Although attempts were made to minimize, as far as possible, leakage from cut tissues, such leakage does occur and must be considered while interpreting the effects of the 5-HT administration *in situ*. In addition to methodological considerations, 5-HT is known to exert its actions both directly on receptors of the vascular smooth muscle and, at least in mammals, indirectly *via* release of endothelial factors such as endothelium-derived relaxing factor (Cocks

and Angus, 1983; Lincoln *et al.* 1990). A relaxation caused by a direct action of 5-HT on the smooth muscle of the jugular vein in rat (Martin *et al.* 1987) and isolated coronary rings from rainbow trout (Small *et al.* 1990) has also been shown. In addition, histochemical evidence points to a possible interaction between serotonergic and adrenergic neurones in the efferent lamellar vessels (Dunel-Erb *et al.* 1989; Bailly *et al.* 1989), and it is likely that differences in the relative activity of these neurones *in situ* and *in vivo* affect the responses to the exogenously added 5-HT. Similarly, 5-HT stored in neurones and/or neuroepithelial cells in the gills has been implicated in sensory mechanisms (Dunel-Erb *et al.* 1982, 1989; Bailly *et al.* 1989; Burleson, 1991; Fritsche and Nilsson, 1992). Thus, 5-HT injected *in vivo* may trigger nervous reflexes similar to those seen during hypoxia, and such effects may be unrelated to the direct responses observed *in situ*.

In contrast to the situation in mammals, where the gastrin/CCK-like peptides increase gut blood flow (Rozsa *et al.* 1985; Holm-Rutili and Berglindh, 1986), CCK-8 and caerulein are known to cause vasoconstriction in the gut of the cod (Jönsson *et al.* 1987). In the present study, CCK-8 and caerulein produce marked branchial vasoconstriction in both types of experiments. *In vivo*, the peptides reduced cardiac output, although f_H remained constant or even increased (see Fig. 4). The reduced stroke volume is probably a result of an increased end-systolic volume of the heart caused by the marked increase in ventral aortic blood pressure. The experiments show that gastrin/CCK-like peptides, which have been demonstrated by immunohistochemistry in neurones within the gills (S. Holm-gren, unpublished observations), may form part of the branchial vasomotor control system.

In conclusion, we estimate the jugular vein blood flow in the cod to be $1.7\,ml\,min^{-1}\,kg^{-1}$, which corresponds to about 8% of the cardiac output. Adrenaline directed the blood towards the arterio-arterial pathway by vasodila-tation of this pathway and by vasoconstriction of the arterio-venous pathway. 5-HT usually produced marked vasoconstriction in the arterio-arterial pathway of the branchial vasculature in the perfusion experiments. This increase in resistance, together with an increased cardiac output, more than doubled the branchial venous blood flow. CCK-8 and caerulein produced similar cardiovascular effects, including marked constriction of both arterio-venous and, especially, arterio-arterial pathways.

This study establishes the ability of the vascular system of the gills to regulate the branchial venous blood flow and to distribute blood between the arterio-arterial ('respiratory') and the arterio-venous ('nutritive and ionoregulatory') pathways. This regulation will be of importance to the fish in optimizing the respiratory blood flow in relation to water loss in the gills during certain physiological situations, such as exercise.

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