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Adenosinergic and cholinergic control mechanisms during hypoxia in the epaulette shark (*Hemiscyllium ocellatum*), with emphasis on branchial circulation

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Summary

Coral reef platforms may become hypoxic at night during low tide. One animal in that habitat, the epaulette shark (*Hemiscyllium ocellatum*), survives hours of severe hypoxia and at least one hour of anoxia. Here, we examine the branchial effects of severe hypoxia (<0.3 mg oxygen l^{-1} for 20 min in anaesthetized epaulette shark), by measuring ventral and dorsal aortic blood pressure (P_{VA} and P_{DA}), heart rate (f_H), and observing gill microcirculation using epi-illumination microscopy. Hypoxia induced a flow of blood in two parallel blood vessels, termed longitudinal vessels, in the outer borders of the free tip of the gill filament. Hypoxia also induced significant falls in f_H, P_{VA} and P_{DA}, and a biphasic change in ventilation frequency (increase followed by decrease). Adenosine injection (1 μmol kg^{-1}) also initiated blood flow in the longitudinal vessels, in addition to significant drops in P_{VA}, P_{DA} and f_H, and a biphasic response in ventilation frequency (decrease followed by increase) indicating that adenosine influences ventilation. Aminophylline (10 mg kg^{-1}), an A_1 and A_2 adenosine receptor antagonist, blocked the effects of adenosine injection, and also significantly reduced blood flow in the longitudinal vessels during hypoxia. In the second part of the study, we examined the cholinergic influence on the cardiovascular circulation during severe hypoxia (<0.3 mg l^{-1}) using antagonists against muscarinic (atropine 2 mg kg^{-1}) and nicotinic (tubocurarine 5 mg kg^{-1}) receptors. Injection of acetylcholine (ACh; 1 μmol kg^{-1}) into the ventral aorta caused a marked fall in f_H, a large increase in P_{VA}, but small changes in P_{DA} (suggesting increased R_{gill}). Atropine was able to inhibit the branchial vascular responses to ACh but not the hypoxic bradycardia, suggesting the presence of muscarinic receptors on the heart and gill vasculature, and that the hypoxia induced bradycardia is of non-cholinergic origin. The results suggest that adenosine mediates increases in the arterio–venous circulation in the gill during hypoxia. This may serve to increase blood supply to heart and gill tissue.

Key words: elasmobranch, blood pressure, cardiovascular, gill, shunt, bradycardia.

Introduction

The best-studied examples of anoxia-tolerant vertebrates, which include crucian carp (*Carassius carassius*) and some freshwater turtles (genera *Trachemys* and *Chrysemys*), have apparently evolved their anoxia tolerance to allow overwintering at cold temperatures (Lutz et al., 2003). Recently, it has become evident that a very different habitat, coral reefs, may contain numerous hypoxia-tolerant fishes (Nilsson and Østlund-Nilsson, 2004). Some coral reef habitats, such as shallow reef platforms, may become severely hypoxic at night due to the high rate of oxygen consumption of the reef organisms. The coral reef inhabitants include the epaulette shark (*Hemiscyllium ocellatum*), which can survive 3.5 h of severe hypoxia (Wise et al., 1998) and at least 1 h of anoxia at temperatures around 25°C (Renshaw et al., 2002). Although the epaulette shark is the best-studied example of a hypoxia-tolerant reef fish, its respiratory and circulatory response to hypoxia has not been examined.

The anatomy of the elasmobranch gill is somewhat different from that of teleosts (Kempton, 1969; Wright, 1973; Olson and Kent, 1980; Devries and Dejager, 1984). First, the two rows of gill filaments on each gill arch are separated by a septum. Second, the afferent filament artery is not a vessel but a cavernous body [also called medial afferent sinus (Olson and Kent, 1980) or corpus cavernosum (Wright, 1973)] at the free tip of the filament. However, as in teleosts, the branchial circulation of elasmobranchs comprises an arterio–arterial (AA) and arterio–venous (AV) circulation (Cooke, 1980; Olson and Kent, 1980; Devries and Dejager, 1984). Blood

enters the lamellae, the respiratory unit of the gill, through afferent lamellar arterioles from a cavernous body in series with the afferent filament artery (AFA) in the gill filament. After being oxygenated, the blood leaves the lamellae through efferent lamellar arterioles flowing into an efferent filament artery (EFA) and, in the AA circulation, the blood enters the dorsal aorta through the efferent branchial artery. Blood can enter the AV circulation through anastomoses from either the afferent or the efferent side, the precise arrangement may vary from species to species (Randall, 1985; Olson, 2002b).

It was initially suggested that the AV system may serve as a lamellar bypass, hence creating a non-respiratory shunt (Steen and Kruysse, 1964). However, it has become evident that the AV system is a low-pressure system that does not enable blood to re-enter the EFA. The AV circulation enters the branchial veins and is, therefore, returned back to the heart. An increase in the AV circulation has been observed in response to hypoxia in cod, and this may serve to increase the blood supply to the heart and the ion-regulatory cells of the filament epithelium (Sundin et al., 1995).

Most teleost fishes studied respond to hypoxia with an increase in branchial vascular resistance (R_{gill}) (Holeton and Randall, 1967; Petterson and Johansen, 1982; Sundin and Nilsson, 1997), bradycardia and an increase in systemic pressure (Fritsche and Nilsson, 1989; Fritsche and Nilsson, 1990; Bushnell and Brill, 1992; Sundin, 1995). The increase in R_{gill} is possibly related to a cholinergically mediated constriction of a sphincter situated at the EFA near the base of the filament. The site of action of acetylcholine (ACh) in the branchial vasculature was first revealed by Smith (1977), and later confirmed by cardiovascular (Farrell, 1981; Farrell and Smith, 1981) and immunohistochemical (Bailly and Dunel-Erb, 1986) measurements. The increase in R_{gill} caused by a constriction of the sphincter is thought to force more blood into the AV circulation through post-lamellar arterioles (Sundin, 1995). The bradycardia can be inhibited by the muscarinic receptor blocker atropine, revealing that a vagal cholinergic innervation mediates this response. Hypoxic bradycardia by vagal innervation has, for example, been well characterized in dogfish (Taylor and Butler, 1982; Barrett and Taylor, 1985; Taylor, 1994).

There are few studies on the branchial effects of hypoxia in elasmobranchs. Unlike teleosts, which have an atropine-sensitive cholinergic innervation, the increase in gill resistance in nerve-stimulated sharks appears to be entirely due to contraction of striated muscles in the gill arch, because it is abolished by pancuronium, a nicotinic receptor blocker (Metcalfe and Butler, 1984; Chopin and Bennett, 1995). A branchial cholinergic innervation involving muscarinic receptors has, as far as we know, not hitherto been demonstrated in an elasmobranch.

Adenosine is thought to serve an important protective function in hypoxic vertebrates, from mammals to teleost fish (Mubagwa et al., 1996; Lutz et al., 2003). Adenosine is formed from ATP in energy-deficient cells or released during purinergic neurotransmission. In the heart, its negative inotropic and chronotropic effects will reduce oxygen demand, and by inducing coronary vasodilatation, adenosine will increase oxygen supply to the cardiac muscle (Rongen et al., 1997). Moreover, adenosine stimulates glucose uptake in myocardial cells (Wyatt et al., 1989), increasing the substrate for glycolysis.

Very little is known about the effect of adenosine on branchial circulation in fish. There is evidence for the presence of A_1 and A_2 adenosine receptors in the ventral aorta of the spiny dogfish (Evans, 1992). In the blacktip reef shark (*Carcharhinus melanopterus*), adenosine constricts the efferent branchial artery (Bennett, 1996). In rainbow trout, injection of adenosine, and the A_1 receptor agonist CPA caused a 60% reduction of both the AFA and the EFA, and increased R_{gill} with 150% (Sundin and Nilsson, 1996). In the same study, cardiac output ($\dot{Q}$) increased while f_H fell after adenosine injection, indicating an increase in stroke volume (SV).

In the epaulette shark, the only known anoxia-tolerant elasmobranch, we wanted to investigate circulatory and branchial responses to hypoxia. Given the important role of adenosine in hypoxia tolerance, we hypothesised an involvement of adenosinergic mechanisms in the cardiovascular system of this species. A second objective of this study was to characterize cholinergically mediated mechanisms during severe hypoxia.

Materials and methods

Experiments were carried out at Heron Island Research Station, Great Barrier Reef, Australia in April–May. Epaulette sharks (*Hemiscyllium ocellatum* Bonnaterre), weighing 500–1000 g, were captured by hand at low tide on the reef platform, and kept in a 10.000 l tank continuously supplied with sea water (50 l min^{-1}). All sharks were kept in the tank for at least 5 days before any experiments were conducted.

The sharks were anaesthetised in a 50 l tank by adding benzocaine, dissolved in 96% ethanol (50 g l^{-1}) to the water, to a final concentration of 60 mg l^{-1}. After being weighed each fish was placed on a surgical table and was ventilated with aerated seawater floating through a tube inserted into the mouth. This water (25 l containing 30 mg benzocaine l^{-1}) was recirculated in a system held at room temperature (25°C).

An incision was made on the ventral side, posterior to the first gill slit, perpendicular to the midline. To record P_{VA}, the first branchial artery was dissected free and a PE50 polyethylene cannula (Intramedic; Becton Dickinson, Sparks, MD, USA) with a small bubble 10 mm from the tip, was occlusively inserted and secured with a thread around the vessel behind the bubble. The incision was closed by sutures and the cannula was secured to the skin. To record dorsal aortic blood pressure (P_{DA}), the shark was placed on its dorsal side and a PE50 cannula was inserted inside a large needle, which was used as a guide to reach the dorsal aorta by a blind penetration made 30 mm behind the anal opening. The cannula was secured to the skin by sutures. Both cannulas (containing heparin-containing elasmobranch saline: 280 mmol l^{-1} NaCl and 350 mmol l^{-1} urea) were connected to Gould Statham P23 Db

pressure transducers (Gould-Statham, Oxnard, CA, USA). Pressure calibrations were performed each day against a static water column.

During pilot experiments, a Doppler flow probe (20 MHz, 45; Iowa Doppler Products, Iowa City, IA, USA) around the aorta was placed in three individuals to measure $\dot{Q}$. However, in the epaulette shark, the aorta sends off the first branchial arteries directly after the conus arteriosus, so it was impossible to position the Doppler probe without damaging the pericardium. Each time the pericardium was opened, both P_{VA} and P_{DA} fell dramatically during the first 10 min and continued to fall for about 2 h until the shark died. Because of limited access to sharks and placing the Doppler after the first branch would give an incorrect estimate of $\dot{Q}$, no further attempts were made to measure $\dot{Q}$.

To observe microcirculatory changes in the gill, a digital video camcorder (DCR-PC7E; Sony, Japan) was connected to an epi-illumination microscope (Leitz Ortholux; Wetzlar, Germany) fitted with a Leitz Ultropak water immersion objective (22× or 11×) as described by Nilsson et al. (1995) and Sundin (1995).

After surgery, the shark was placed in a plastic chamber and continuously ventilated with seawater (60 l containing 30 mg benzocaine l^{-1}) and left to rest for 1 h before any experiments were conducted. The water level was adjusted with a standpipe to a level that covered the shark. A plexiglas column (1500 mm high, 80 mm diameter) was included in the water circulation. This column was bubbled with N_2 to make the water severelty hypoxic ($[O_2]$<0.3 mg l^{-1}) within 1 min, and adjusted with air to prevent anoxic conditions.

Experimental protocol

First the sharks (*N*=14) were exposed to two 20 min episodes of severe hypoxia to allow microscopic observation of afferent and efferent sides of the filament. After the two initial hypoxic periods, sharks where divided into two groups, one injected with adenosine and the other injected with ACh.

Adenosine

Adenosine was injected into the afferent branchial artery. Cardiovascular measurements and microscopic observations were done continuously on either the afferent or the efferent side of the gill filament (*N*=7). After 30 min, the adenosine injection was repeated and the gill filament was observed on the opposite side. Before the experiments, we tested for suitable doses and 1 μmol adenosine kg^{-1} was chosen. Subsequently, the non-specific adenosine receptor agonist aminophylline (10 mg kg^{-1}) was injected, followed by two more hypoxic periods and measurements (as above). The experiments ended with adenosine injections to verify an efficient adenosine receptor blockade.

ACh

For ACh (Sigma; St Louis, MO, USA) a similar protocol as for adenosine was used. ACh was injected twice into the ventral aorta (*N*=7) and cardiovascular measurements and microscopic observations were done on the afferent and the efferent side. Continuous measurements after each treatment were made until the parameters were had returned to pre-injection or pre-hypoxic levels. Before the experiments, we tested for suitable doses and 1 μmol ACh kg^{-1} was chosen. Next the animals were treated with atropine (2 mg kg^{-1}; Sigma). After 30 min, the ACh injections were repeated to ensure that all muscarinic receptors were blocked. The injections were subsequently followed by two additional hypoxic periods. Finally, 5 mg kg^{-1} of the nicotinic receptor antagonist tubocurarine (Sigma) was injected and the two hypoxic periods and ACh injections were repeated.

Data treatment and statistics

Cardiovascular variables were sampled using Labview (version 5.0, National Instruments, Austin, TX, USA). Sampling frequency used was 30 Hz and mean values were subsequently calculated at 30 s (hypoxia) and 15 s (adenosine and ACh) intervals. Data are presented as means ± S.E.M. In Fig. 1, only data for every 1 min 30 s are shown to allow separation of the S.E.M. From the pulsed blood pressure signals, f_H was derived using a Labview-based calculation program. Evaluations of statistically significant differences were done with Grap Pad Instat (3.01; Graph Pad Software, San Diego, CA, USA) and a repeated measure analysis of variance (ANOVA) was performed to detect statistically different changes during the hypoxic and drug injection periods. A Dunnet post-test was used to evaluate which time interval was significantly different from pre-injection values. In the figures, significant differences (P<0.05) over a time interval are indicated with a line. To detect significant differences between non-treated (control) and treated (aminophylline, atropine and tubocurarine) sharks, the control values at each time point of a cardiovascular variable in each animal were subtracted from the sum of the treatment values, and a repeated measure ANOVA was performed to detect significant differences and indicated in the figures with an asterisk. A Student's *t*-test was used on the last pre-hypoxic value to detect differences between groups before and after treatment with aminophylline. In the duplicate exposures (hypoxia, ACh and adenosine) only the first set of pressure and f_H data were used for statistical analysis.

Measurements of microvascular changes in the branchial vasculature were done on a TV screen using a slide calliper for EFA diameter measurements and a stopwatch to detect difference in the velocity of erythrocytes between different exposures. The pre-exposure (hypoxia and drug injected) values were set to 100% and differences were compared using a non-parametric ANOVA (Freidmann's test) followed by Dunn post-test.

A Fisher's exact test was used to evaluate differences in blood flow in the longitudinal vessels in the filament between treated and non-treated individuals. Fig. 4 shows number of sharks with blood flow as a proportion of the total number of sharks at each time point. Ventilation frequency was measured with a stopwatch, and a repeated measure ANOVA was

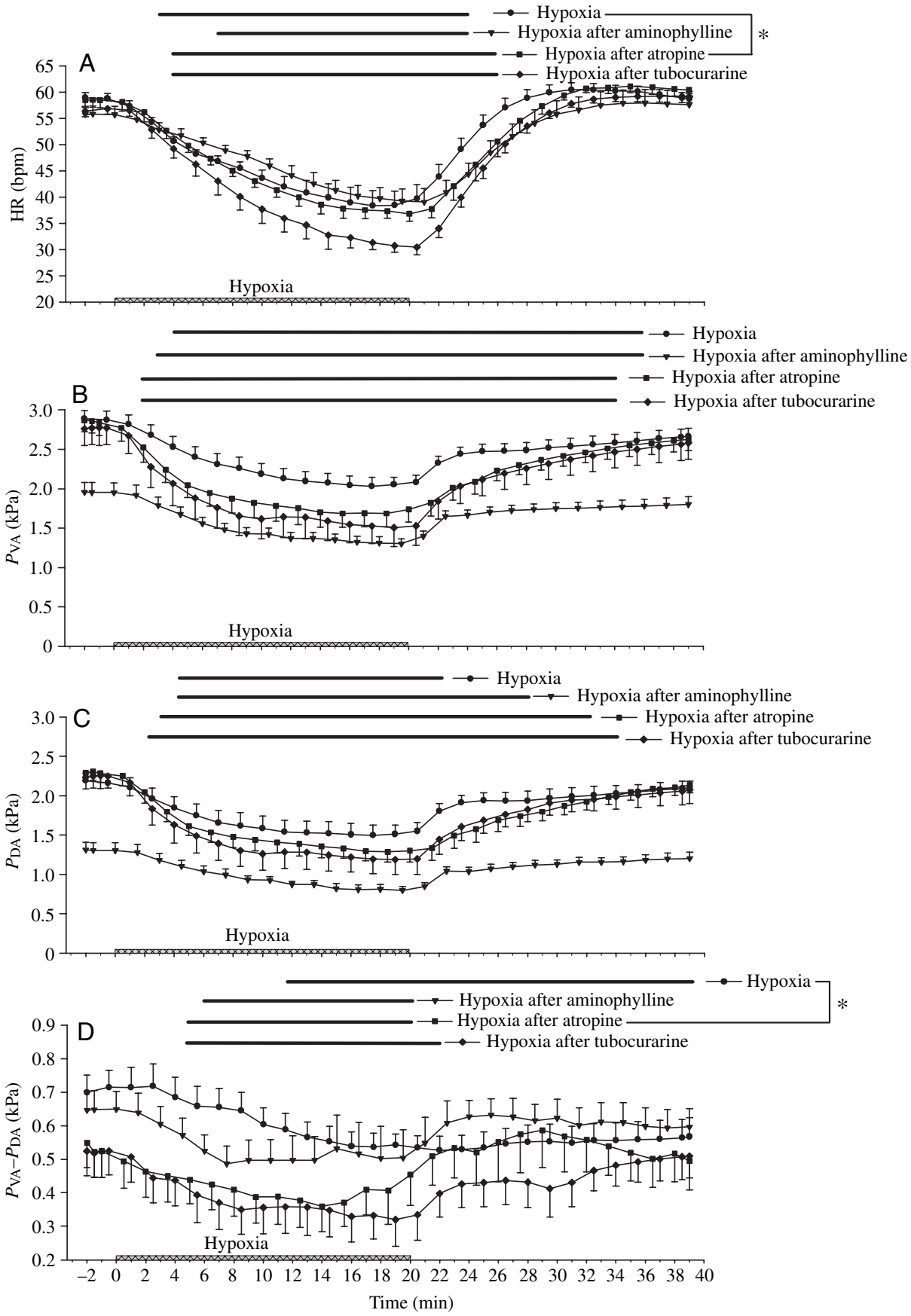
A
Hypoxia
Hypoxia after aminophylline
Hypoxia after atropine
Hypoxia after tubocurarine
*
HR (bpm)
Hypoxia
B
P_{VA} (kPa)
C
P_{DA} (kPa)
D
$P_{VA}-P_{DA}$ (kPa)
Time (min)

Fig. 1

Fig. 1. Effects of 20 min of severe hypoxia (0.3 mg l^{-1}), indicated by the bar on *x*-axis. (A) Heart rate (f_H), (B) ventral aortic blood pressure (P_{VA}), (C) dorsal aortic blood pressure (P_{DA}) in control (●, *N*=14) aminophylline-treated (▼, *N*=7), atropine-treated (■, *N*=7) and tubocurarine-treated (◆, *N*=7) sharks. All values are means ± S.E.M. Time-dependent changes were tested using repeated measures ANOVA with Dunnet post-test. Lines indicate the time periods that differ significantly from the last normoxic value (P<0.05). Differences in the effects of hypoxia between aminophylline-treated and control sharks (A) and between hypoxia- and atropine-treated (D) are indicated by an asterisk.

performed to detect significant differences. All post-exposure values highlighted in the results are minimum or maximum values.

Results

As shown in Fig. 1A, 20 min of hypoxia caused a steady decrease in f_H from 58.44±0.97 beats min^{-1} to 39.03±2.61 beats min^{-1} (*N*=14, P<0.05). Simultaneously, P_{VA} and P_{DA} fell from 2.85±0.11 kPa and 2.14±0.09 kPa to 2.04±0.19 kPa and 1.50±0.07 kPa, respectively (*N*=14, P<0.05; Fig. 1B,C). The *in vivo* microscopy indicated a 24.79±3.84% decrease in the velocity of the erythrocytes in the EFA (*N*=12, P<0.05; Fig. 2A). There was no reduction in the diameter of the EFA in hypoxia (data not shown). On the afferent side, there was no distinct AFA in the free tip of the filament but a continuous cavernous body formed a network of afferent vessels. After 4–5 min into the hypoxic period, blood appeared in openings in the vasculature between the bases of adjacent lamellae (white arrows in Fig. 3) and blood flowing through these openings emptied into (and thereby unmasked) two blood vessels running in parallel with the outer border of the filament (outlined in Fig. 3). The blood flow direction was towards the base of the filament (black arrows in Fig. 3). In some individuals, the flow was slightly pulsatile, but in most cases it was uniform. These longitudinal vessels went all the way from the filament base to the tip, where the free tip attaches to the septum. About 12–15 min into the hypoxic period the blood flow in the vessels ceased (Fig. 4A). Using higher magnification the openings appeared still to be open, and the start and stop of blood flow may have been due to vasodilatation and vasoconstriction, respectively, of the connecting vessel deeper down in the filament.

The anaesthetised shark displayed ventilatory movements, although they were shallower than in awake sharks. The ventilation frequency initially increased and then decreased during hypoxia (Fig. 5A).

Treatment with aminophylline (10 mg kg^{-1}) had a large effect both on P_{VA} and on P_{DA} resting values, which were significantly reduced from 2.82±0.12 kPa to 1.95±0.17 kPa, and from 2.06±0.09 kPa to 1.3±0.09 kPa respectively (P<0.05; Fig. 1B,C). There was no significant reduction in f_H after aminophylline treatment, but there

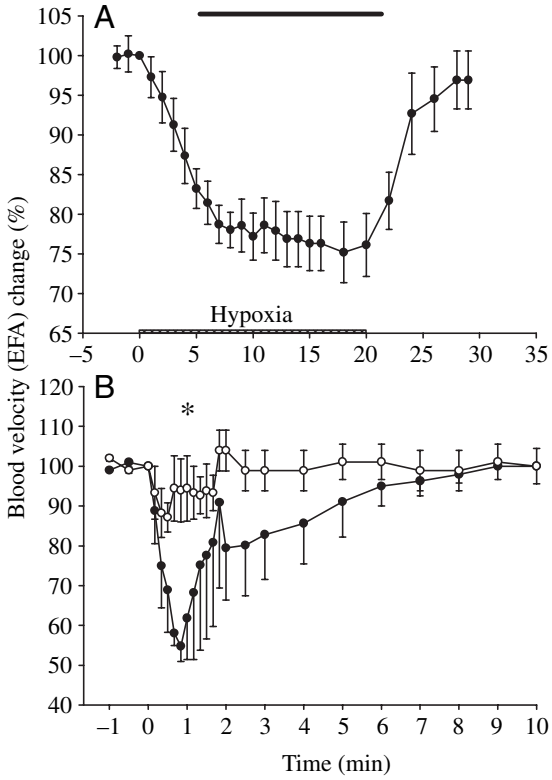

Fig. 2. Effects of (A) hypoxia (*N*=12) and (B) acetylcholine (ACh; 1 μmol kg^{-1}) injections (*N*=7) on blood flow velocity in efferent filament arteries (EFA). ●, ACh; ○, ACh after atropine injection. Values are means ± S.E.M. and normalized to the proportion (%) of pre-hypoxic velocity. The line indicates a time period significantly different from the last pre-exposure value [P<0.05; non-parametric ANOVA (Freidmann test) with Dunn post-test]. The asterisk indicates a significant difference between atropine treatment and control.

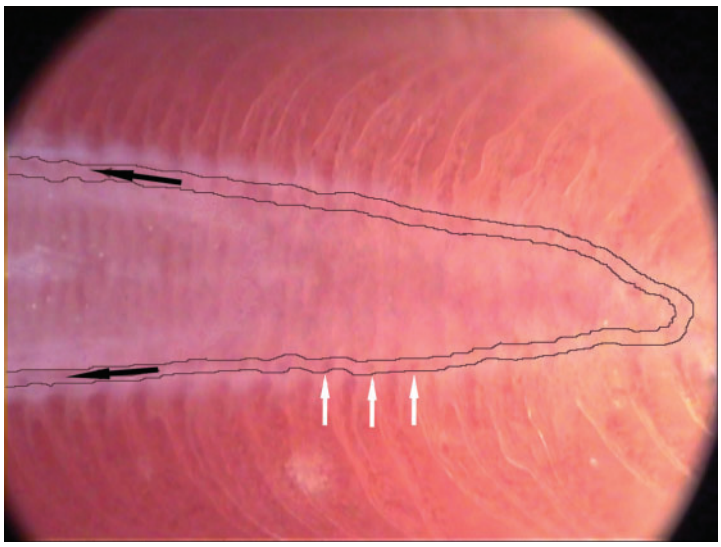

Fig. 3. Video micrograph of the free tip of a filament showing longitudinal vessel during hypoxia. The vessel is outlined with a black line, and direction of blood flow is indicated by black arrows. White arrows point toward anastomoses where the blood started flowing during hypoxia and adenosine injections. Scale bar, 100 μm.

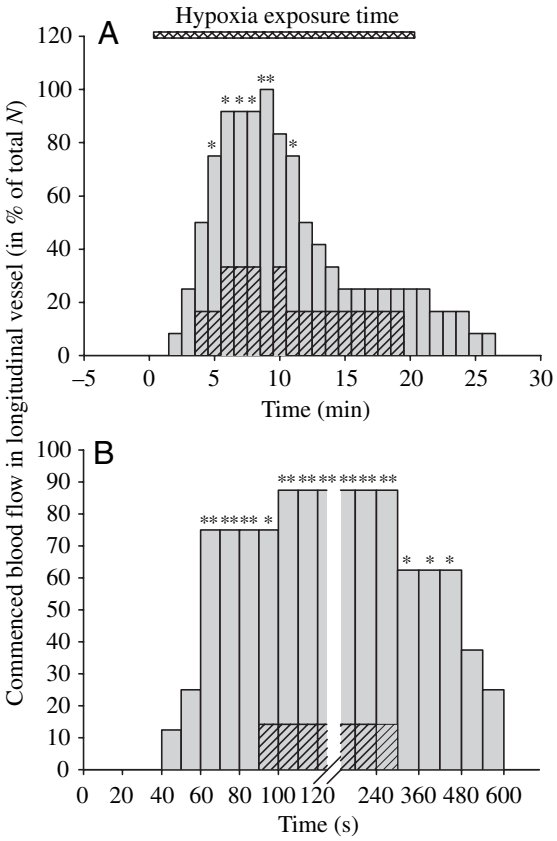

Fig. 4. Frequency distribution of sharks displaying commenced blood flow in the longitudinal vessels during (A) hypoxia (grey bars; N=12) and (B) adenosine injection (1 μmol kg^{-1}) (grey bars; N=8), and after aminophylline treatment during (A) hypoxia (hatched bars; N=6) and (B) adenosine injections (1 μmol kg^{-1}) (hatched bars; N=7). Injection of adenosine at time 0. An asterisk indicates a significant difference between aminophylline treated and control group (*P<0.05, **P<0.01) (Fisher's exact test).

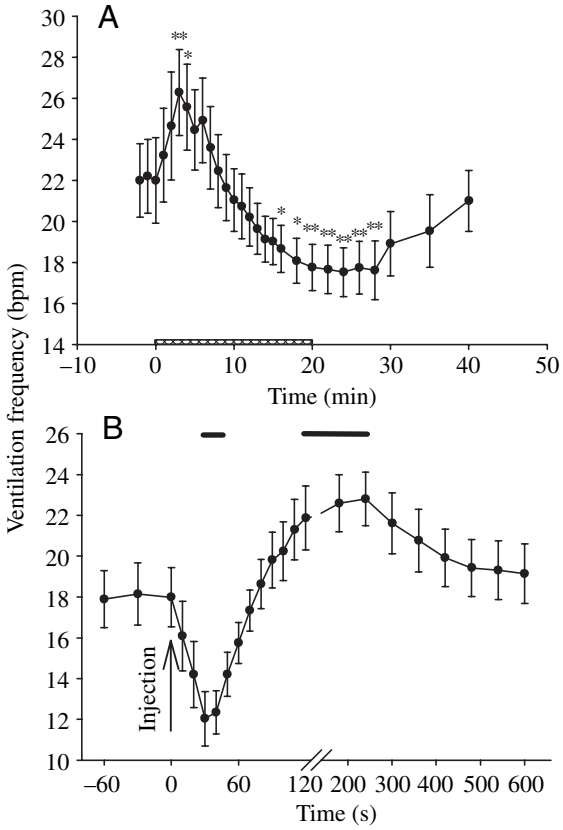

Fig. 5. Effects of (A) hypoxia (N=9) and (B) adenosine injections (N=7) (1 μmol kg^{-1}) on ventilation frequency. Line indicates significant time interval different from last normoxic value; non-parametric ANOVA (Freidman test) with Dunn post-test. Values are mean ± S.E.M.

was a significant difference indicated by the ANOVA only, between aminophylline-treated and non-treated sharks during hypoxia (although the post-test failed to detect differences at specific time points). The initial reduction in f_H appeared to be slower after aminophylline treatment, but f_H decreased more towards the end of the hypoxic period after aminophylline treatment. Even if blood pressures were lower in aminophylline-treated animals, they showed the same response pattern as in control animals during hypoxia, with significant reductions in P_{VA}, P_{DA} and f_H (Fig. 1). There was no significant difference between aminophylline-treated and non-treated sharks in P_{VA} or P_{DA} responses to hypoxia.

After aminophylline injections, only two out of seven hypoxic sharks showed a commenced blood flow in the longitudinal vessels (Fig. 4A). Aminophylline abolished the spontaneous ventilatory movements in the shark.

In the second group of sharks, injection of atropine (2 mg kg^{-1}) had neither effects on normoxic f_H and blood pressures, nor on the hypoxia induced bradycardia and reduced blood pressures (Fig. 1). Similarly, atropine did not inhibit the commenced blood flow into the longitudinal vessels and the biphasic change (an initial increase followed by a decrease) in ventilatory frequency observed during hypoxia (data not shown). In fact, the only significant effect of atropine on the hypoxic responses was a reduction in the pressure difference (P_{VA}–P_{DA}) over the gills (Fig. 1D; P<0.05).

When adenosine was injected, f_H fell from 58.60±1.10 beats min^{-1} to 48.87±4.49 beats min^{-1} (P<0.05; Fig. 6A). Simultaneously, P_{VA} fell from 2.73±0.13 kPa to 2.12±0.11 kPa (P<0.05; Fig. 6B) and P_{DA} from 2.01±0.08 kPa to 1.39±0.11 kPa (P<0.05; Fig. 6C). Thus, adenosine injections mimicked the hypoxic cardiovascular response patterns. There was no change in the pressure fall over the gills (P_{VA}–P_{DA}; Fig. 6D). Adenosine also initiated blood flow in the longitudinal blood vessels (Fig. 4B). Most of the responses to injected adenosine were abolished after aminophylline treatment, but adenosine still caused a significant reduction in blood pressures and f_H. The post-test revealed a significant difference between treated and non-treated sharks in P_{VA} between 60 s and 720 s, and in P_{DA} between 30 s and 300 s (P<0.05).

Adenosine also induced a significant and biphasic response in ventilation frequency with an initial increase, followed by

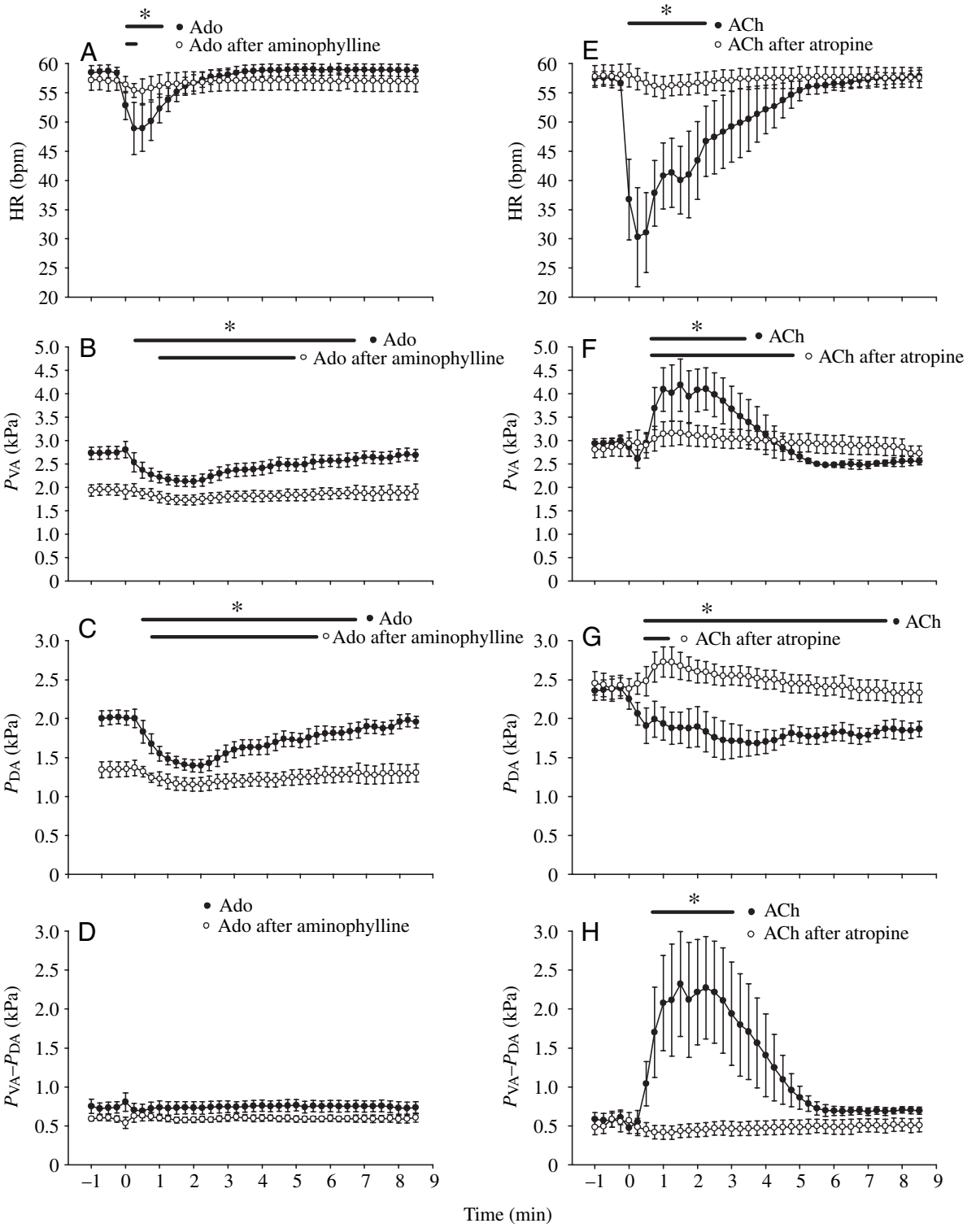

Fig. 6. Cardiovascular responses to adenosine (Ado) injections (at time zero) on (A) heart rate (f_H), (B) ventral aortic blood pressure (P_{VA}) and (C) dorsal aortic blood pressure (P_{DA}) in control (●, N=7) and aminophylline-treated (○, N=6) sharks. All values are mean ± S.E.M. Time-dependent changes were tested using repeated measures ANOVA with Dunnet post-test. Lines indicate the time periods that differ significantly from the last normoxic value (P<0.05). A significant ANOVA between aminophylline-treated and control sharks in response to adenosine injections is indicated by an asterisk.

a decrease (Fig. 5B), which was opposite to the pattern elicited by hypoxia (Fig. 5A). In aminophylline treated sharks, adenosine injection did not initiate breathing movements again.

After injecting 1 μmol kg^{-1} ACh into normoxic sharks, f_H fell from 58.18±0.82 beats min^{-1} to 29.93±6.91 beats min^{-1} within 30 s (Fig. 6E; P<0.05). There was also an increase in P_{VA} from 2.84±0.13 kPa to 4.13±0.41 kPa (Fig. 6F) and a decrease in P_{DA} from 2.25±0.18 kPa to 1.72±0.17 kPa (Fig. 6G; P<0.05) resulting in a large pressure fall (P_{VA}–P_{DA}) over the gills (Fig. 6H). Visual observations of the branchial vasculature in the microscope detected no changes in the diameter of the EFA after ACh injections. However, the speed of the erythrocytes in the EFA was significantly reduced by 46.7±4.2% (P<0.05) during the first minute after the injection (Fig. 3). Blood flow in the longitudinal vessels was commenced by ACh injection in three out of six individuals (data not shown). Another interesting observation was that in the animals where ACh transiently paused the heart beat, the thickness of the filament appeared to decrease, and simultaneously the filament bent downwards. This supports the idea that the cavernous body functions as a supportive hydrostatic skeleton in the free tip of the shark gill filament (Devries and Dejager, 1984). Although small significant increases in P_{VA} and P_{DA} occurred after atropine treatment (Fig. 6F,G), atropine blocked the ACh-induced increase in pressure difference over the gills (P_{VA}–P_{DA}; Fig. 6H). Atropine inhibited the commenced blood flow in the longitudinal vessels and the reduced speed of erythrocytes in the EFA (Fig. 2B).

Injection of tubocurarine in atropine treated animals had no significant effect on any of the cardiovascular values measured in either normoxic or hypoxic conditions (Fig. 1). By contrast, the spontaneous ventilatory movements disappeared after injection of tubocurarine verifying a successful inhibition of nicotinic receptors.

Discussion

Gill blood flow patterns in hypoxia

Hypoxia initiated blood flow in two longitudinal blood vessels in the filament of the epaulette shark. In normoxia, we could observe small vascular openings between each lamella, but no apparent vessel leading from these holes. However, about 5 min into hypoxia, blood started flowing out from these openings, unmasking a previously invisible vessel at the outer edge of the filament. The blood flow went from the tip towards the base of the filament. This hypoxia-induced flow of blood ended by itself about 15 min into the hypoxic period and did not start again during recovery to normoxic conditions. Interestingly, this initiation of blood flow could be blocked by aminophylline, suggesting a dependence on adrenergic receptors.

The longitudinal vessels are probably the same vessels as those described in spiny dogfish as vessels draining blood from interlamellar vessels (Olson and Kent, 1980; Devries and Dejager, 1984). The interlamellar vessels form a sheet of vessels that run parallel to and between the inner margins of the lamellae. The blood supply for the intralamellar vessels varies from species to species. Most fishes appear to have post-lamellar anastomoses (Olson, 2002a,b). However, pre-lamellar anastomoses exists in eel (Hughes et al., 1982) and in spiny dogfish, they are also present (Olson and Kent, 1980), but not in the smallspotted catshark (Dunel and Laurent, 1980; Randall, 1985). The longitudinal vessels in the spiny dogfish are connected with the intralamellar vessels system and display a marked constriction as they pass between afferent lamellar arterioles (Devries and Dejager, 1984). The control mechanisms of these anastomoses have been unknown, but the present results suggest that they are under adenosinergic control. The intralamellar sinus is in series with the diaphragmal sinus, which drains into the anterior cardinal vein and jugular vein, thereby returning blood directly to the heart (Devries and Dejager, 1984). The portion of $\dot{Q}$ that normally enters the AV circulation varies between species, but has been estimated to 8% in Atlantic cod (*Gadus morhua*; Sundin and Nilsson, 1992), 7% in trout (*Oncorhynchus mykiss*; Ishimatsu et al., 1988), and up to 30% in eel (Hughes et al., 1982). A full understanding of the AV system is lacking. However, this circulation is probably important for the many functions of the gill tissue, including ion-balance, acid–base regulation and metabolism of circulating hormones (Laurent, 1984; Olson, 1991, 1996, 2002a; Ishimatsu et al., 1992; Goss et al., 1998; Randall and Brauner, 1998). Our observation of a hypoxia-induced flow in the longitudinal vessels suggests an increased drainage into the AV circulation. Thus, this may be a mechanism for increasing the supply of blood to non-respiratory functions of the gills during energy deficiency. The fact that this blood is delivered directly back to the heart, may also function to protect the heart during hypoxia.

Hypoxic bradycardia

Hypoxia induced a profound bradycardia, and a decrease in P_{VA} and P_{DA}, in the epaulette shark. Unfortunately, we were not able to measure $\dot{Q}$ directly because of difficulties in attaching a Doppler flow probe without damaging the pericardium, but a reduction in blood flow velocity in the EFA was observed during hypoxia indicating a fall in $\dot{Q}$. A reduced $\dot{Q}$ during hypoxia has, in fact, been observed in two dogfish species, *Scyliorhinus stellaris* (Piiper et al., 1970) and spiny dogfish (Davie and Farrell, 1991). Thus, it is possible that P_{VA} and P_{DA} fell during hypoxia because of a reduced $\dot{Q}$ (Fig. 2A). There was no significant change in the blood pressure fall over the gills. Both an increase and decrease in R_{gill} during hypoxia has been observed in elasmobranchs (Satchell, 1962; Piiper et al., 1970; Butler and Taylor, 1971; Butler and Taylor, 1975; Kent and Peirce, 1978). The increase in R_{gill} displayed by hypoxic teleosts may result in lamellar recruitment and also serve to increase the overflow to the AV circulation (Sundin, 1995).

Interestingly, the hypoxic bradycardia in the epaulette shark could not be blocked by the cholinergic muscarinic-receptor antagonist atropine (Fig. 1A). In this respect, the epaulette shark differs from other elasmobranchs and teleosts exposed to hypoxia, which invariably show an atropine-sensitive

reduction in f_H. Because ACh injections into the ventral aorta of the epaulette shark reduced f_H by 50%, and as this response was abolished by atropine treatment, it proves that this species indeed has muscarinic receptors on the heart, but these are apparently not stimulated by nervous release of ACh during hypoxia.

Other neurotransmitters, in addition to ACh, have been found to elicit a negative chronotropic effect during electrical stimulation of the vagus nerve in the toad, *Bufo marinus* (Courtice and Delaney, 1994). Cardiac nerve endings in this toad contain ACh, somatostatin and galanin, which all are co-released upon nerve stimulation. However, there seems to be no chronotropic effect of other substances than ACh in the shark *Heterodontus portusjacksoni* (Preston and Courtice, 1995), making it less likely that neuropeptides are mediating bradycardia in the epaulette shark. The slow onset of the hypoxia-induced bradycardia in the epaulette shark also argues for a non-nervous origin of the response. In the epaulette shark, the bradycardia was not fully developed until after 15 min, while in spiny dogfish (*Scyliorhinus canicula*), atropine-blockable bradycardia sets in within 1 min of hypoxia (Taylor et al., 1977).

In teleosts, acidosis reduces $\dot{Q}$ by suppressing both f_H and SV (Farrell and Jones, 1992; Driedzic and Gesser, 1994). Although no measurement of blood pH has been done in the epaulette shark, it is likely that the sevenfold increase in lactate observed at low oxygen tensions in this shark (Routley et al., 2002) acidifies the blood. This may be one of the reasons for the observed reduction in f_H in the epaulette shark. In sea raven (*Hemitripterus americanus*) and the ocean pout (*Macrozoarces americanus*), a reduction in blood pH from 7.9 to 7.4 reduced $\dot{Q}$ by 12% and 18%, respectively, by suppressing both SV and f_H (Farrell et al., 1983).

Interestingly, an α-adrenoceptor-mediated negative chronotropy has been observed in the spiny dogfish (Capra and Satchell, 1977) and we can not rule out this mechanism underlying the hypoxia-induced bradycardia. However, 30 min of hypoxia did not elevate the plasma catecholamines in the spiny dogfish (Perry and Gilmour, 1996).

Effects of ACh

Upon ACh injections, the blood flow came almost to a complete stop in the EFA between heartbeats. Still no constriction of the EFA was observed in the outer portion of the filament, which strongly suggests a constriction further down the EFA, possibly at the EFA sphincter. By contrast, marked vasoconstrictions could be observed in the distal portions of AFA and EFA in rainbow trout (Sundin and Nilsson, 1997). Thus, it appears that muscarinic receptors are not spread throughout the epaulette shark gill vasculature, but may be concentrated more basally or in the EFA sphincter. ACh injections ultimately lead to a large increase in P_{VA}, whereas P_{DA} remained unchanged. Taken together, this indicates a substantial increase in R_{gill}. Efferent branchial artery (EBA) rings from blacktip reef shark and the lemon shark (*Negaprion queenslandicus*) have been found to constrict in response to ACh in an atropine-sensitive manner (Bennett, 1993, 1996). It is possible that also the observed increase in R_{gill} in the epaulette shark could be related to constriction of the EBA.

ACh injections induced blood flow in the longitudinal vessels in the filament tip in three out of six individuals. Although an adenosinergic mechanism is probably involved (see below), it is possible that also the large increase in R_{gill} (induced by ACh) 'forces' more blood into the AV system. As hypothesized by Randall (1982), expansion of the elastic high-pressure vessels during increased branchial pressure probably squeezes the extensively valved low-pressure system, augmenting flow in the AV vessels. Thus, an adenosine-induced dilation of the AV system in the epaulette shark (see below) may work in concert with a cholinergic increase in branchial pressure to increase oxygenated blood supply to the gill tissues during hypoxia. In addition, when a larger portion of $\dot{Q}$ enters the AV circulation, the heart muscle will receive more oxygenated venous return in addition to the blood supplied by the coronary system.

Effects of adenosine

Adenosine injections mimicked the hypoxic responses in the epaulette shark by reducing f_H, P_{VA} and P_{DA} (Fig. 6A–C). Interestingly, adenosine also induced blood flow in the longitudinal vessels. Most of the effect was blocked by the unspecific adenosine receptor antagonist aminophylline. In mammals, adenosine has a cardio-protective role during hypoxia (Mubagwa et al., 1996), and it is possible that it also has such a function in other vertebrates. In rainbow trout, adenosine has negative chronotropic and inotropic effects (Aho and Vornanen, 2002). There is evidence for the presence of A_1 and A_2 receptors in the ventral aorta of spiny dogfish (Evans, 1992), and adenosine has negative inotropic and chronotropic effects in smallspotted catshark (Meghji and Burnstock, 1984).

Adenosine constricts the branchial circulation in teleosts (Colin and Leray, 1981; Sundin and Nilsson, 1996; Sundin et al., 1999). Using epi-illuminating microscopy, Sundin and Nilsson (1996) observed a 60% reduction of the EFA diameter in rainbow trout, probably mediated by the A_1 receptor, because the response was mimicked by the specific A_1 agonist CPA. However, utilizing the same technique we could not observe any constriction in the EFA in the epaulette shark after adenosine injections.

Interestingly, adenosine initiated a flow of blood in the longitudinal vessels. This observation, together with the finding that aminophylline could block the same blood flow during hypoxia, makes adenosine a likely candidate for mediating this hypoxic response. A likely scenario is that adenosine, as a result of hypoxic energy deficiency, functions to reduce f_H and possibly to increase blood flow directly from the gill back to the heart. Unlike other fishes, the reduction in f_H is not mediated by muscarinic receptors and must, therefore, be mediated by some other mechanism acting on the heart. Interestingly, the ability of adenosine to reduce the activity of adenylyl cyclase through A_1 receptors and to activate an

outward potassium current $I_{K–Ado}$ appear to be sufficient to explain the negative chronotropic and inotropic effects of adenosine in mammals (Shryock and Belardinelli, 1997). However, aminophylline did not inhibit the hypoxic bradycardia, indicating that adenosine is not involved in this response in the epaulette shark. The epaulette shark may have a large adenosinergic tone on blood vessels because aminophylline injections significantly reduced P_{VA} and P_{DA}, indicating a general vasodilatation. However, as we do not have a measurement of $\dot{Q}$, we cannot rule out a reduction in *SV*, because f_H was not affected in aminophylline-treated normoxic animals. However, removal of a possible positive adenosinergic tone on *SV* by injecting aminophylline is not likely, because we observed a negative chronotropic effect of injecting adenosine in the epaulette shark.

During the experiments, the epaulette shark displayed shallow rhythmic ventilatory movements that showed an initial increase, followed by a ventilatory depression, in response to hypoxia. A similar response has been reported previously in un-anaesthetized epaulette sharks, although at a higher frequency (Routley et al., 2002). The respiratory rate we recorded was most likely lower because of the anaesthesia, but because the same response pattern was observed, the mechanisms are probably similar. Interestingly, a reversed biphasic response pattern, an initial decrease followed by an increase, was observed when adenosine where injected (Fig. 5). The ventilatory movement ceased when aminophylline was injected either suggesting that adenosine is important for the respiratory drive, or that unspecific effects of aminophylline interferes with respiratory functions. An increase in ventilation frequency as a response to hypoxia is widespread among fishes but, to our knowledge, this is the first indication of an involvement of adenosine in a respiratory reflex in fish. In rat, injection of adenosine into the circulation inhibits respiration through vagal pulmonary C fibres (Kwong et al., 1998). As adenosine levels are likely to increase during hypoxia, a role of adenosine in ventilatory control in fish during hypoxia needs further attention.

Conclusion

Taken together, our results indicate a role for adenosine in the hypoxic survival of the epaulette shark. Adenosinergic control of blood flow into the longitudinal vessels is likely to be involved in increasing in the portion of $\dot{Q}$ that flows through the AV circulation during hypoxia. This could be a mechanism aimed at increasing blood supply to heart and gill tissue during hypoxic challenges. Another major finding is that despite the presence of muscarinic receptors on the heart, the epaulette shark responds to hypoxia with a large and highly unusual atropine insensitive bradycardia. Because the elasmobranchs belong to an evolutionary 'old' line in vertebrate evolution, and as this shark can survive several hours of severe hypoxia at high temperatures, it appears to be a good model for revealing evolutionary conserved mechanisms in hypoxic survival.

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Abbreviations

AA	arterio–arterial
ACh	acetylcholine
AFA	afferent filament artery in the gill filament
AV	arterio–venous
EBA	efferent branchial artery
EFA	efferent filament artery in the gill filament
f_H	heart rate (beats min^{-1})
P_{DA}	dorsal aortic blood pressure
P_{VA}	ventral aortic blood pressure
$\dot{Q}$	cardiac output
R_{gill}	branchial vascular resistance
SV	stroke volume

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