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STORAGE AND RELEASE OF CATECHOLAMINES FROM THE CHROMAFFIN TISSUE OF THE ATLANTIC HAGFISH *MYXINE GLUTINOSA*

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Summary

A variety of *in vivo* and *in situ* experiments were performed on the Atlantic hagfish (*Myxine glutinosa*) (i) to characterize the levels of circulating catecholamines during acute stresses, including hypoxia, anoxia or physical disturbance (air-exposure), and (ii) to evaluate the potential mechanisms of catecholamine release from the major sites of storage, the systemic heart and posterior cardinal vein (PCV).

Adrenaline and noradrenaline were stored at roughly equivalent concentrations (approximately 20 $\mu g g^{-1}$) in cardiac tissue, whereas noradrenaline was the predominant catecholamine stored in the PCV (approximately 50 $\mu g g^{-1}$). The heart stored larger quantities of total catecholamines than did the PCV (approximately three times greater) owing to its larger mass and higher concentration of adrenaline.

Exposure of chronically cannulated hagfish to acute hypoxia [mean water P_{O_2} (Pw_{O_2})=1.4kPa; 10.5mmHg) for 30min caused a significant decrease in arterial P_{O_2} (from 11.5±1.3kPa to 1.2±0.3kPa) and arterial O_2 content (from 3.9±0.3ml100ml^{-1} to 0.9±0.2ml100ml^{-1}). The hypoxaemia was associated with a significant increase in plasma noradrenaline levels, whereas plasma adrenaline levels were unaffected. Exposure of uncannulated fish to anoxia (Pw_{O_2} approximately 0kPa) or physical disturbance (15min of air-exposure) also elicited pronounced increases in plasma noradrenaline levels (6–10 times) and, to a lesser extent, adrenaline levels (2–3 times).

An *in situ* saline-perfused heart preparation was utilized in an attempt to elucidate the mechanism(s) underlying the stress-induced release of catecholamines from the chromaffin tissue of the heart and PCV. *Non-specific* cell membrane depolarization using 40 or 60mmoll^{-1} K^+ in the saline elicited a marked release of catecholamines, confirming the suitability of the preparation to assess *specific* physiological mechanisms of catecholamine release. Lower concentrations of K^+ (15–20mmoll^{-1}) did not evoke

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catecholamine release, indicating that relatively minor elevation in plasma [K^+], as might occur during hypoxia, is not a contributing factor. The cholinergic receptor agonist carbachol (10^{-5}–10^{-4} mol kg^{-1}) caused a significant release of catecholamines, yet the likelihood of a similar mechanism operating *in vivo* is doubtful because the hagfish heart is not thought to be innervated. Simulation of (i) internal hypoxaemia by perfusing with anoxic saline or (ii) physical disturbance by perfusing with relatively acidic saline (pH approximately 7.0) failed to elicit catecholamine release. Further, the elevation of perfusion (input) pressure to simulate a rise in venous blood pressure, as might occur during hypoxia or physical disturbance, was also without effect on release. The addition of pituitary extract (from Atlantic cod, *Gadus morhua*) to the inflowing saline caused a marked release of catecholamines from the chromaffin tissue.

Thus, the mechanism(s) of release of catecholamines from the heart of hagfish during stress *in vivo* remains unclear, although preliminary experiments suggest the possible involvement of pituitary hormones.

Introduction

The principal component of the acute stress response in fish is the release of the catecholamines, adrenaline and noradrenaline, from chromaffin tissue (see review by Randall and Perry, 1992). The accompanying elevation of circulating catecholamine levels initiates a series of compensatory processes to ameliorate the effects of stress on physiological function (Perry and Wood, 1989; Thomas and Perry, 1992; Randall and Perry, 1992; Randall, 1990; Jensen, 1991; Nikinmaa, 1992). In particular, the elevated catecholamine levels serve to enhance branchial O_2 transfer and blood O_2 transport by influencing cardiovascular (Fritsche and Nilsson, 1993), ventilatory (see Randall and Taylor, 1991; Perry *et al.* 1992 for opposing views), red blood cell (Nikinmaa, 1992) and spleen (Perry and Kinkead, 1989) function.

The storage and release of catecholamines have been extensively studied in the teleosts. In these fishes, the chromaffin cells are contained predominantly within the walls of the posterior cardinal vein as well as being scattered throughout the tissue of the anterior (head) kidney (see Nilsson, 1983; Randall and Perry, 1992). The chromaffin cells are innervated by preganglionic cholinergic fibres of the sympathetic nervous system (Nilsson, 1976). In Atlantic cod (*Gadus morhua*), electrical stimulation of these fibres (Nilsson *et al.* 1976), to elicit endogenous neurotransmitter release, or exogenous application of the cholinergic neurotransmitter acetylcholine (Perry *et al.* 1991) causes release of the stored catecholamines. Consequently, the predominant mechanism of catecholamine mobilization during stress *in vivo* is thought to be through neuronal stimulation of the chromaffin cells (Randall and Perry, 1992). In addition, it is likely that changes in blood chemistry, including oxygen depletion (Perry *et al.* 1991) or elevated [K^+] (Opdyke *et al.* 1983), may directly cause the chromaffin cells to release catecholamines.

The cyclostomes (hagfish and lampreys), like the dipnoans, are unusual among the vertebrates because large quantities of catecholamine-containing chromaffin cells are located within the heart (Augustinsson *et al.* 1956; Bloom *et al.* 1961, 1962; Johnels and Palmgren, 1960; Ostlund *et al.* 1960; von Euler and Fange, 1961) in addition to those

found within the great veins. Further, the heart of hagfish is devoid of extrinsic neuronal innervation (e.g. Augustinsson *et al.* 1956; Jensen, 1961, 1965). Thus, a significant pathway of catecholamine release, neuronal stimulation of the chromaffin cells, is presumably absent in hagfish. There is indirect evidence, however, to suggest that catecholamines are released into the circulation of hagfish during acute hypoxia (Axelsson *et al.* 1990; Forster *et al.* 1991), although direct measurements of plasma catecholamines are lacking.

The goals of the present study were twofold; the first was to provide direct evidence that the Atlantic hagfish (*Myxine glutinosa*) does indeed release stored catecholamines into the circulation during stress. The second goal was to elucidate the underlying mechanisms promoting the release of catecholamines from the posterior cardinal vein (PCV) and the aneuronal heart. This was accomplished using an *in situ* saline-perfused PCV/heart preparation.

Materials and methods

Experimental animals

Atlantic hagfish (*Myxine glutinosa* L.) weighing between 40 and 120g (experimental *N*=171) were caught in wooden traps lowered onto the floor of the Gullmarn fjord in the vicinity of Kristineberg Marine Biological Station (Fiskebackskil, Sweden). They were maintained indoors in aquaria supplied with flowing fjord sea water (temperature 10–11˚C) where they were allowed to feed *ad libitum* on rotting mackerel. The aquaria were kept in a dark corner of the room in order to minimize the potential disturbance of ambient light. The fish were allowed to acclimate to these conditions for at least 2 weeks prior to experimentation (June–July 1992).

Atlantic cod (*Gadus morhua* L.) weighing approximately 800g (experimental *N*=8) were provided by local fishermen and maintained outdoors in large fibreglass aquaria supplied with flowing sea water. The fish were not fed and were used within 2 weeks of capture.

In vivo *experiments*

Hagfish were anaesthetized in a solution of $0.4\,g\,l^{-1}$ ethyl *m*-aminobenzoate (MS222, Sigma). After approximately 20min, the fish were transferred to an operating table where the same anaesthetic solution was continuously passed over the gills *via* a tube placed into the mouth. The dorsal aorta was exposed by making a lateral incision approximately 4cm from the tip of the tail. A polyethylene cannula (Clay Adams PE 50) was stretched to reduce its diameter and inserted anteriorly into the dorsal aorta and secured with ligatures. Fish were transferred to opaque tubes immersed in acrylic boxes. The tubes were sealed at each end and were provided with flowing aerated sea water; the cannula left the tube through a slot. Fish were allowed to recover for at least 24h prior to experimentation. The cannulae were flushed daily with heparinized ($50\,i.u.ml^{-1}$ sodium heparin) sea water.

Series 1. Catecholamine storage levels

Fish (*N*=9) were removed from their aquaria and decapitated. The heart (atria and

ventricle combined) and posterior cardinal vein (PCV) were removed, placed in microcentrifuge tubes, and frozen in liquid N_2. The cardiac tissue and PCV were weighed and then homogenized (using a hand-held homogenizer) separately in 2ml of 4% perchloric acid (PCA) containing 0.5mgml^{-1} sodium bisulphate. The resultant suspension was centrifuged (10000***g*** for 2min) and a sample (20 μl) of the supernatant was diluted 50 times with 4% PCA. The extracts were frozen in liquid N_2 and then stored at −80°C prior to analysis of catecholamines (within 2 weeks).

Series 2. The effects of hypoxia on blood respiratory variables and plasma catecholamines

Separate groups of fish (N=6–9) were first exposed to normoxia ($P_{W_{O_2}}$=19.7–19.8kPa) and then either maintained further under normoxia (controls) or exposed to severe environmental hypoxia ($P_{W_{O_2}}$=1.4kPa) for a 30min period. Arterial blood samples (0.6ml) were withdrawn from the dorsal aortic cannula immediately prior to hypoxic exposure (termed Pre) and after 30min of hypoxia (termed Post). Blood samples were immediately analyzed for total oxygen content ($C_{a_{O_2}}$), arterial oxygen partial pressure P_{O_2} ($P_{a_{O_2}}$), haemoglobin concentration ([Hb]), pH (pHa) and haematocrit (Hct). The remaining blood was centrifuged (10000***g*** for 2min) and the plasma added to microcentrifuge tubes containing 20 μl of 0.2mol l^{-1} glutathione and 0.2mol l^{-1} EGTA. The plasma samples were frozen in liquid N_2 and then stored at −80°C prior to analysis of catecholamine levels (within 3 months).

Hypoxia was initiated by recirculating a static volume (approximately 30l) of chilled (10°C) sea water, gassed with N_2, to the holding tubes. The P_{O_2} of the inflowing water ($P_{W_{O_2}}$) was continuously monitored (see below) and, if necessary, the rate of N_2 delivery to the system was adjusted accordingly. In practice, $P_{W_{O_2}}$ was lowered to 1.4kPa after 5 min of hypoxia and thereafter remained constant.

Series 3. The effects of acute anoxia or physical disturbance on plasma catecholamine levels

These experiments were designed to evaluate the effect of stress on plasma catecholamine levels. They were performed on uncannulated animals owing to the relatively poor success rate using cannulated fish. Hagfish were placed into plastic barrels (approximately 15l volume) and allowed to adjust to these conditions for 3h. Anoxia was induced by vigorous gassing of the water with N_2. Using this protocol, $P_{W_{O_2}}$ was lowered to near 0kPa within 5–10min. The fish (N=7) were removed after 30min and a blood sample (0.5–1.0ml) was quickly taken (within 10s) by puncture of the subcutaneous sinus.

Separate experiments were performed to evaluate the impact of (i) the handling/blood sampling itself, and (ii) the vigorous bubbling of the barrel. To evaluate the effects of handling/blood sampling, fish (N=8) were removed from their holding aquarium and a blood sample was taken immediately from the subcutaneous sinus. To evaluate the effects of the potential stress associated with the bubbling of the barrel, fish (N=6) were placed into a barrel that was aerated vigorously for 30min and then subjected to blood sampling as above.

In a final series of experiments, fish (N=8) were removed from their aquarium and exposed to the air for 15min, at which time a blood sample was taken from the subcutaneous sinus. In all cases, plasma was obtained by centrifugation and stored at −80°C prior to analysis of catecholamine levels.

In vitro *experiments – oxygen equilibrium curves*

Blood was obtained by subcutaneous puncture (see above) and pooled to yield a volume of approximately 5ml (adequate for one O_2 equilibrium curve); typically 6–7 fish were required to generate sufficient blood. The Hct was increased to 20% by resuspending the blood in homologous plasma so as to allow unbiased comparisons between curves and to increase the accuracy and precision of the total O_2 determinations. The blood was heparinized (50i.u.ml^{-1}) and allowed to sit on ice for 3h prior to use. Oxygen equilibrium curves were constructed by equilibrating blood samples (0.4ml) with 80, 5, 3, 2 or 1% O_2 in a Radiometer BMS2 tonometer. Four separate curves were constructed using 0.2, 0.3, 0.4 and 0.5% CO_2. The gas mixtures were provided by gas-mixing pumps (Wösthoff). After equilibration for 20min, each sample was analyzed for O_2 content and pH. The amount of O_2 specifically bound to haemoglobin (Hb) at any given P_{O_2} was determined by subtracting from the total O_2 content the amount of O_2 physically dissolved in the plasma using values of O_2 solubility in trout plasma (Boutilier *et al.* 1984). O_2 equilibrium curves were constructed by plotting the amount of O_2 specifically bound to Hb against P_{O_2}. P_{50} values (P_{O_2} at which Hb is half saturated with O_2) were calculated from Hill plots [log[y/(1−y)] *versus* logP_{O_2}]. Values for the percentage of Hb O_2-saturation (y) were calculated assuming that the blood equilibrated with 80% O_2 was 100% saturated with O_2.

In situ *experiments*

Hagfish were anaesthetized in a solution of 0.4 g l^{-1} MS222 in sea water. After 20–30min, fish were injected *via* the subcutaneous sinus with 1500i.u. of heparin (0.3ml). A ventral incision was made to expose the posterior cardinal vein, systemic heart and ventral aorta. The posterior cardinal vein and ventral aorta were cannulated (PE 90 tubing) for the inflow and outflow of perfusion fluid, respectively. The fish was removed from the anaesthetic solution, immersed in sea water without anaesthetic and the heart was perfused with aerated hagfish saline (pH8.1) (Axelsson *et al.* 1990) at ambient water temperature. All fish remained stationary throughout the experimental period. The systemic heart was perfused from a constant-pressure reservoir that was held 6cm above the level of the heart (except in experiments designed to evaluate the effects of elevated perfusion pressure). Owing to the resistance of the tubing and blood vessel, the actual perfusion pressure was probably considerably less than 0.6kPa (6cmH_2O). Although the perfusion pressure was probably greater than venous blood pressure *in vivo* (Satchell, 1986), it was required to attain sufficient volumes of outflowing perfusion fluid (1–1.5ml min^{-1}). The heart was perfused for 20min before sample collection. After this period of stabilization, effluent perfusion fluid was collected in microcentrifuge tubes containing 20 μl of glutathione/EGTA (as above) at 1min intervals, which was usually sufficient to obtain the required 1.0–1.5ml of fluid. The actual volume of effluent

perfusion fluid collected over the 1min interval was not determined so it was not possible to calculate the *rate* of release of catecholamines (i.e. molmin^{-1}). Instead, the concentration of catecholamines in the effluent perfusion fluid was used as an index of release. Only in those instances where a particular treatment markedly affected effluent flow (see below) would the two methods yield discrepant conclusions. The fish used in the *in situ* experiments were roughly of a uniform size (40–50g) so the data were not normalized for mass differences among the animals.

The collected samples were frozen in liquid N_2 and stored at −80°C prior to analysis of catecholamine content. In all experiments, two Pre samples were collected, followed by five Post samples after a given experimental intervention (see below). In general, this intervention lasted 1–2min and, thus, in control (N=8) experiments, the five Post samples were collected after a 2min waiting period. In addition to the control experiments, six separate series of experiments were performed.

Series 1. The effects of elevated [K^+] on catecholamine release

The heart was perfused for 2min with hagfish saline containing the usual [K^+] of 8 mmol l^{-1}. The [K^+] in the saline was then abruptly increased (using KCl) to 15, 20, 40 or 60mmol l^{-1} (N=8 for each K^+ concentration) by switching perfusate reservoirs. After 2 min, samples were collected for an additional 5min. The highest levels of K^+ used (40 and 60mmol l^{-1}) frequently caused the heart to slow or beat erratically, although it was still possible to collect adequate volumes of perfusion fluid for catecholamine analysis.

Series 2. The effects of carbachol on catecholamine release

After the 2min control period, a bolus injection of 10^{-5} molkg^{-1} (N=6) or 10^{-4} molkg^{-1} (N=8) (in 0.2ml of saline) of the cholinergic receptor agonist carbachol was administered to the preparation *via* a valve in the inflow catheter.

Series 3. The effects of local hypoxia on catecholamine release

Reservoirs of hagfish saline and sea water were pre-equilibrated with N_2. After the 2 min control period, the sea water was drained from the bath holding the fish and replaced with hypoxic sea water ($P_{W_{O_2}}$=2.3±0.3kPa; N=8). The heart was then perfused with hypoxic saline ($P_{W_{O_2}}$=0.7±0.3kPa; N=8) by switching perfusion reservoirs. The $P_{W_{O_2}}$ of the sea water covering the exposed cardiac tissue was maintained at approximately 2kPa for the duration of the experiment by bubbling with N_2.

Series 4. The effects of acidosis on catecholamine release

The heart was perfused for 2min with hagfish saline of the usual pH (8.1) The pH in the saline was then abruptly decreased (using HCl) to 7.1±0.03 (N=6) by switching perfusate reservoirs. After 2min, samples were collected for an additional 5min.

Series 5. The effects of elevated perfusion (input) pressure on catecholamine release

Under control conditions the perfusion pressure was maintained at 0.6kPa (6cmH_2O). In order to assess the potential impact of elevated venous return pressure on catecholamine release, the perfusate reservoir was raised to 1.7kPa (17cmH_2O) (N=6) or

2.9kPa (30cmH_2O) (*N*=8). In these experiments, the effects of elevated pressure on cardiac frequency were also evaluated.

Series 6. The effect of pituitary extracts on catecholamine release

Pituitary extracts were prepared from Atlantic cod as follows. Fish were killed by a blow to the head. The brain was exposed and the entire pituitary was removed and placed in ice-cold cod saline ([NaCl]=150.1mmol l^{-1}, [KCl]=5.1mmol l^{-1}, [$MgSO_4.7H_2O$] and [$CaCl_2.7H_2O$]=1.9mmol l^{-1}, [$NaH_2PO_4.2H_2O$]=1.9mmol l^{-1}, [$NaHCO_3$]=7.0mmol l^{-1} and [glucose]=1 g l^{-1}; modified from Holmgren and Nilsson, 1974). Three pituitaries were pooled and homogenized in 2ml of cod saline using a hand-held glass homogenizer. The resultant suspension was centrifuged (10000 ***g*** for 2min) and the supernatant was removed and frozen at −80°C until required.

Perfused preparations (*N*=6) were given bolus injections of 300 μl of extract, which was roughly equivalent to half of a pituitary.

In a separate series, pituitary extracts were prepared (*N*=5) as above and assessed for catecholamine levels.

Analytical procedures

Blood, water and saline P_{O_2} and pH were measured using Radiometer P_{O_2} and microcapillary pH electrodes in conjunction with Radiometer PHM-73 acid–base analyzers and BMS2 blood microsystems, respectively. All electrodes were maintained at ambient water temperatures. Blood O_2 content was measured on 20 μl samples using the method of Tucker (1967) at 37°C. Blood [Hb] was determined spectrophotometrically using a commercial kit (Sigma).

Noradrenaline and adrenaline levels were determined on alumina-extracted tissue extracts and on plasma or saline samples (200 μl) using high performance liquid chromatography with electrochemical detection (Woodward, 1982); methyl dopamine was used as the internal standard.

Statistical analyses

All data are presented as mean ±1 standard error of the mean (S.E.). For comparison of two means, paired or unpaired Student's *t*-tests were used, where appropriate. For multiple comparisons, the data were analyzed by one-way analysis of variance followed by Fisher's LSD multiple-comparison test. In all cases, 5% was taken as the fiducial limit of significance.

Results

In vivo *experiments*

Series 1. Catecholamine storage levels

The catecholamines adrenaline and noradrenaline were stored at roughly equal concentrations in the heart (21.3±3.3 and 20.4±4.1 μg g^{-1}, respectively; Fig. 1A). In contrast, the PCV contained predominantly noradrenaline (49.4±8.8 μg g^{-1}) with only low levels of adrenaline being detected (1.9±0.9 μg g^{-1}; Fig. 1A). Owing to the greater mass

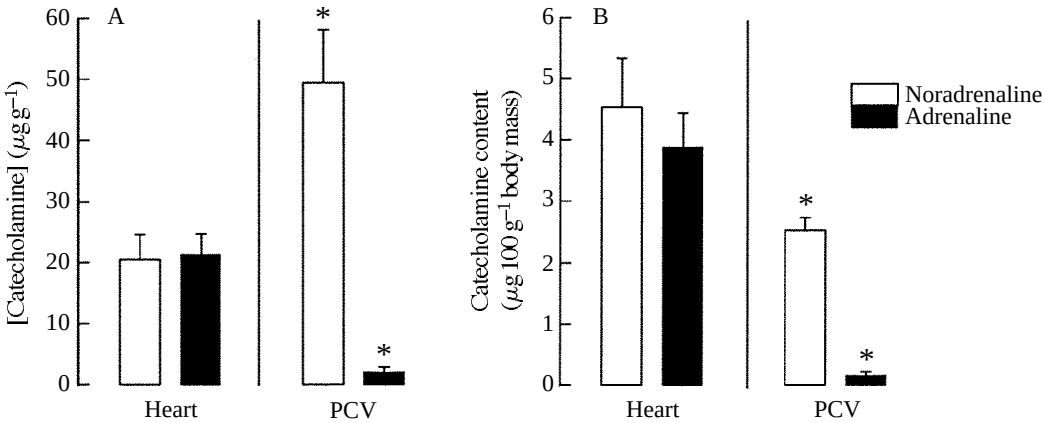

Fig. 1. The levels of stored catecholamines in the heart (atria and ventricle) and posterior cardinal vein (PCV) of hagfish expressed either on the basis of (A) concentration ($\mu g\,g^{-1}$) or (B) total content ($\mu g\,100\,g^{-1}$ bodymass). Values shown are means +1 S.E.; * indicates a significant difference from the corresponding value in the heart.

of the heart (approximately 10 times greater) and its higher concentration of adrenaline, the cardiac tissue stored greater quantities of catecholamines than the PCV (Fig. 1B).

Series 2. The effects of hypoxia on blood respiratory variables and plasma catecholamines

The exposure of fish to acute hypoxia (P_{WO_2}=1.4±0.1kPa) for 30min caused pronounced reductions in P_{aO_2}, C_{aO_2} and $[O_2]$/[Hb] (Table 1). [Hb] and Hct were also reduced, but this was probably a result of blood sampling alone, because similar changes were observed in the control fish. Arterial blood pH remained constant during hypoxia. Although the hagfish were maintained in opaque tubes, it was nevertheless apparent from the movements of the cannulae leaving the tubes that the fish became agitated during the hypoxic period. Plasma [noradrenaline] was elevated during hypoxia (from 3.16±0.7 to 10.76±2.4nmol l^{-1}), whereas adrenaline levels were unchanged (Table 1).

Series 3. The effects of acute anoxia or physical disturbance on plasma catecholamine levels

The sampling of blood by subcutaneous puncture or the vigorous aeration of the holding water had no appreciable effect on plasma catecholamine levels (Fig. 2). However, exposure of the fish to anoxic conditions for 30min or physical disturbance by air-exposure caused marked increases in plasma noradrenaline levels and, to a lesser extent, adrenaline levels (Fig. 2).

In vitro *experiments – O_2 equilibrium curves*

Representative Hill plots at four CO_2 levels (0.2–0.5% CO_2) are shown in Fig. 3A. In each instance, the slope of the Hill plot (Hill coefficient; n_H) was approximately equal to 1 with the mean n_H for the four curves being 1.03±0.01. The P_{50} values varied between

Table 1. *The effects of acute hypoxia on arterial blood respiratory variables and plasma catecholamine concentrations*

	Control fish (*N*=5)		Hypoxic fish (*N*=9)	
	Pre	Post	Pre	Post
$P_{W_{O_2}}$ (kPa)	19.8±0.1	19.6±0.2	19.7±0.1	1.4±0.1*
$P_{a_{O_2}}$ (kPa)	10.8±1.3	8.4±1.1	11.5±1.3	1.2±0.3*
$C_{a_{O_2}}$ (ml100ml^{-1})	3.8±0.7	3.0±0.6	3.9±0.3	0.9±0.2*
pHa	7.91±0.10	7.85±0.12	7.96±0.04	7.84±0.02
[Haemoglobin] (g100ml^{-1})	4.1±0.5	3.2±0.7*	3.8±0.4	2.9±0.4*
Haematocrit (%)	11.2±0.7	9.5±0.9*	11.7±0.8	9.8±1.0*
[O_2]/[haemoglobin] (nl g^{-1})	0.93±0.14	0.94±0.15	1.06±0.18	0.30±0.09*
[Adrenaline] (nmol l^{-1})	0.36±0.1	1.01±0.5	1.20±0.4	1.43±0.5
[Noradrenaline] (nmol l^{-1})	1.8±0.4	1.5±0.9	3.2±0.7	10.8±2.4*

Blood samples were withdrawn before (Pre) and after (Post) 30min of exposure to water P_{O_2} ($P_{W_{O_2}}$) ranging between 1.1 and 1.6kPa.

The control fish were subjected to continuous normoxic exposure. Values shown are means ± 1 S.E.; * indicates a significant difference from the Pre value (P<0.05; paired t-test).

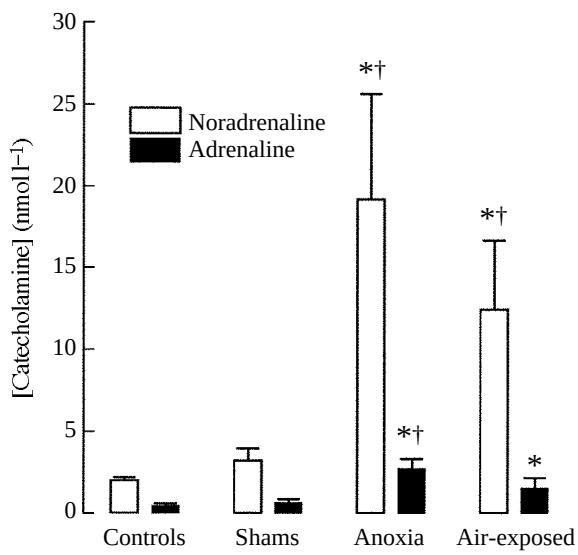

Fig. 2. The effects of acute stresses, including anoxia ($P_{W_{O_2}}$ approximately 0kPa) or air-exposure (15min) on plasma catecholamine levels measured by subcutaneous puncture of uncannulated hagfish. The control fish were removed from their holding aquaria; the sham fish were placed into buckets and subjected to vigorous aeration. Values shown are means +1 S.E.; * indicates a significant difference from the control fish; † indicates a significant difference from the sham fish.

1.71kPa (0.2% CO_2; pH7.93) and 3.0kPa (0.5% CO_2; pH7.67), indicating a pronounced CO_2-induced Bohr effect; the Bohr factor was calculated to be −1.01 (Fig. 3B). There was no obvious Root effect (data not shown).

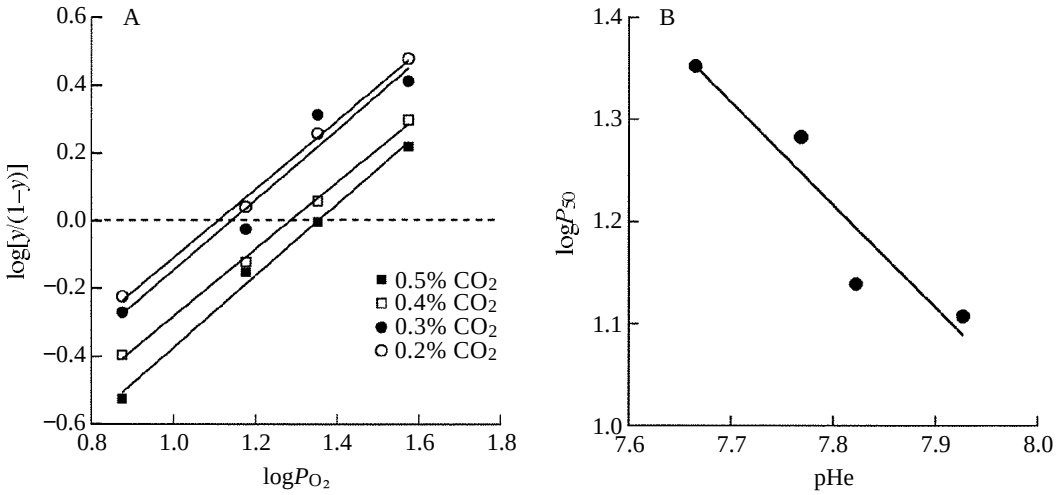

Fig. 3. (A) Hill plots of *in vitro* O_2 equilibrium curves of hagfish whole blood (Hct 20%) equilibrated with 0.2–0.5% CO_2. The P_{50} values were 1.71kPa (0.2% CO_2, pHe7.927), 1.84kPa (0.3% CO_2, pHe7.832), 2.53kPa (0.4% CO_2, pHe7.768) and 3.0kPa (0.5% CO_2, pHe7.665). The mean Hill coefficient (n_H) was 1.03±0.01. (B) The relationship between extracellular pH (pHe) and logP_{50} in hagfish whole blood. The Bohr factor was calculated to be −1.01. See text for further details.

In situ *experiments*

The saline-perfused heart/PCV preparation released the catecholamines adrenaline and noradrenaline equally at a basal rate, although the quantities released were low (Fig. 4A). Acute elevation of the [K^+] in the saline from 8mmoll^{-1} to 15 or 20mmoll^{-1} did not affect catecholamine release (Fig. 4B,C), whereas raising [K^+] to 40 or 60mmoll^{-1} stimulated the release of both catecholamines (Fig. 4D,E). The effect of 40mmoll^{-1} K^+ was short-lived, but the effect of 60mmoll^{-1} K^+ persisted for the duration of the sampling period.

The bolus injection of 10^{-5} or 10^{-4}molkg^{-1} carbachol caused transient dose-dependent increases in the release of catecholamines (Fig. 5); noradrenaline was the predominant catecholamine released.

None of the treatments designed to simulate the potential stress-induced changes *in vivo*, including perfusing with hypoxic or acidotic saline or elevating perfusion pressure, affected catecholamine release from the heart/PCV (Table 2). The elevation of perfusion pressure from 0.6 to 2.9kPa caused pronounced increases in cardiac frequency (from 38±1.9 to 43±1.4beatsmin^{-1}), as previously reported (Jensen, 1961); the elevation of input pressure from 0.6 to 1.7kPa was without effect.

The application of pituitary extracts derived from Atlantic cod caused pronounced and equivalent stimulation of adrenaline and noradrenaline release (Fig. 6). The effect of pituitary extracts was not an artefact related to contamination of the extract with catecholamines because the extract contained relatively low levels of catecholamines ([adrenaline]=0.40±0.2nmoll^{-1}; [noradrenaline]=7.0±5.9nmoll^{-1}).

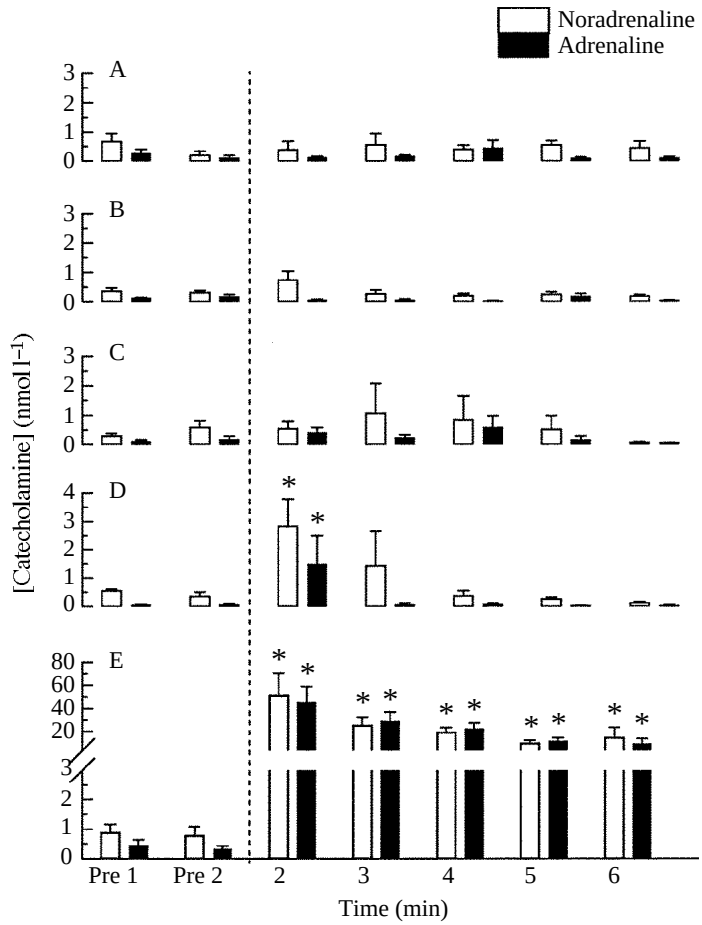

Fig. 4. Outflowing perfusate catecholamine levels in an *in situ* saline-perfused heart preparation during (A) control conditions or after the addition of (B) 15mmol l^{-1} K^+, (C) 20mmol l^{-1} K^+, (D) 40mmol l^{-1} K^+ or (E) 60mmol l^{-1} K^+. The dashed line indicates the acute elevation of [K^+], after which a waiting period of 2min ensued before sampling recommenced. Values shown are means +1 S.E.; * indicates a significant difference from the value immediately preceding the addition of K^+ (Pre 2). Note the different scales for the *y*-axis in D and E.

Discussion

In vivo *experiments*

The results of the present study provide the first direct evidence to our knowledge that plasma catecholamine levels are elevated in hagfish during acute stress. Earlier unpublished work by M. Mazeaud (see Table 6.2 in Nilsson, 1983) demonstrated stress-induced release of catecholamines in another cyclostome, the sea lamprey (*Petromyzon marinus*). Previous studies on the Atlantic hagfish *Myxine glutinosa* (Axelsson *et al.* 1990) or the New Zealand hagfish *Eptatretus cirrhatus* (Forster *et al.* 1991, 1992) indirectly suggested that catecholamines were released into the circulation during

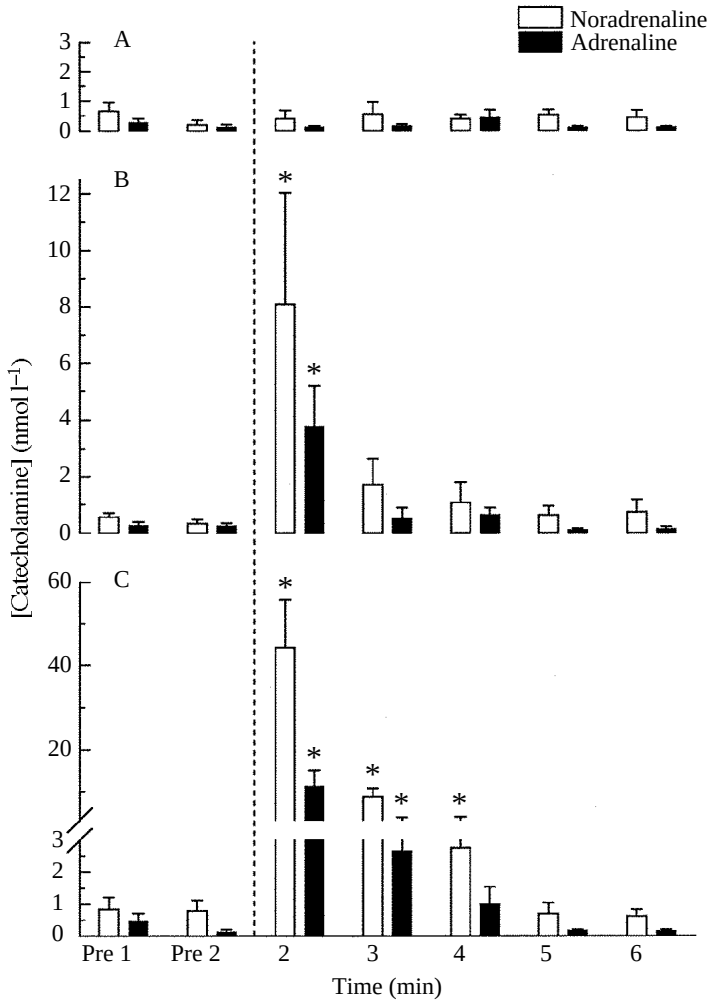

Fig. 5. Outflowing perfusate catecholamine levels in an *in situ* saline-perfused heart preparation during (A) control conditions or after the addition of (B) 10^{-5} molkg^{-1} carbachol or (C) 10^{-4} molkg^{-1} carbachol. The dashed line indicates the acute elevation of carbachol, after which a waiting period of 2min ensued before sampling recommenced. Values shown are means +1 S.E.; * indicates a significant difference from the value immediately preceding the addition of carbachol (Pre 2). Note the different scales for the *y*-axes.

hypoxia. The basis for this assumption was the observation of increased blood pressure in *Myxine* and *Eptatretus* (Axelsson *et al.* 1990; Forster *et al.* 1992) and the increase in branchial vascular resistance in *Eptatretus* (Forster *et al.* 1992) that accompanied the hypoxia. Owing to the reputed absence of adrenergic autonomic nerve fibres innervating the vasculature of hagfish (see Nilsson, 1983), it was suggested that these effects were caused by circulating catecholamines. The finding of significantly elevated noradrenaline levels in the plasma of hypoxic hagfish in the present study supports the contention that humoral catecholamines are involved in the control of cardiovascular function in this

Table 2. *The effects of anoxia, acidosis (pH7.0) or elevated input (perfusion) pressure (*P*i) on catecholamine release from the* in situ *saline-perfused heart of hagfish*

	[Catecholamine] (nmol l^{-1})	
	Pre	Post
Control (8)	1.20±0.28	1.14±0.23
Anoxia (8)	2.14±0.80	2.41±0.98
Acidosis (6)	1.47±0.56	1.87±0.63
Elevated *P*i (1.7kPa) (6)	1.63±0.66	1.27±0.37
Elevated *P*i (2.9kPa) (8)	1.88±0.39	1.70±0.45

For simplicity, the data are presented as total catecholamine (adrenaline plus noradrenaline) levels immediately before (Pre) and 2min after (Post) changing the experimental conditions.

Means ± 1 S.E.; *N* values are indicated in parentheses.

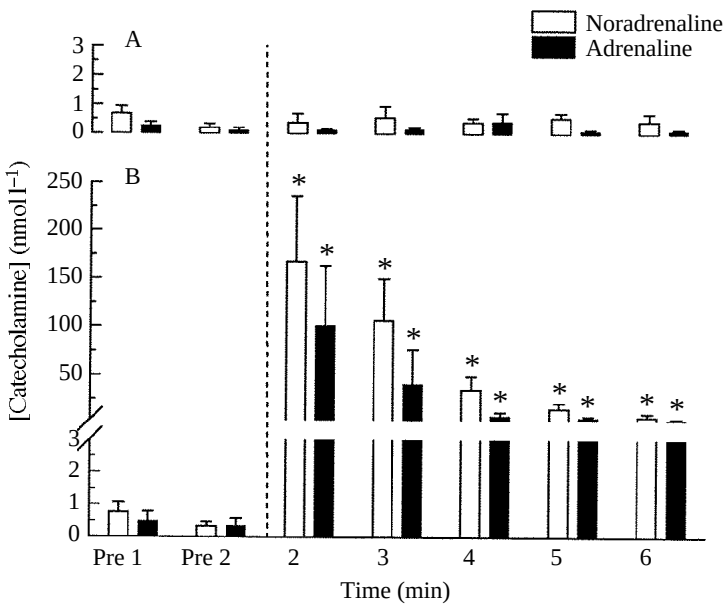

Fig. 6. Outflowing perfusate catecholamine levels in an *in situ* saline-perfused heart preparation during (A) control conditions or (B) after addition of pituitary extracts from Atlantic cod (*Gadus morhua*). The dashed line indicates the acute addition of pituitary extract, after which a waiting period of 2min ensued before sampling re-commenced. Values shown are means +1 S.E.; * indicates a significant difference from the value immediately preceding the addition of extract (Pre 2). Note the different scales for the *y*-axes.

group of fish. The reliance of the hagfish on circulating catecholamines for regulating cardiovascular function resembles the strategy utilized by elasmobranchs (Butler and Metcalfe, 1988) in which the innervation of the heart and blood vessels (branchial and systemic) by adrenergic autonomic nerve fibres is poorly developed or absent altogether (Nilsson, 1983). The involvement of circulating catecholamines is probably less

important in the teleosts (see review by Fritsche and Nilsson, 1993) owing to well-developed innervation of the vasculature and cardiac tissue by adrenergic autonomic nerve fibres.

In addition to the potential effects of circulating catecholamines on the systemic or branchial blood vessels, it is conceivable that the release of endogenous catecholamines stored within the heart may directly affect cardiac contractility. Axelsson *et al.* (1990) reported that treatment of *Myxine* with the β-adrenoceptor antagonist sotalol significantly lowered cardiac frequency, suggesting tonic adrenergic control of the heart. Axelsson *et al.* (1990) noted a significant increase in cardiac frequency (f_H) in *Myxine* after intravascular injection of adrenaline, yet during hypoxia f_H remained constant. The absence of hypoxic cardioacceleration in that study indicates that the level of hypoxia may not have been severe enough to elicit sufficient catecholamine mobilization to affect the heart [the $P_{W_{O_2}}$ in the study of Axelsson *et al.* (1990) was 1.5–2.2kPa compared with a mean $P_{W_{O_2}}$ of 1.4kPa in the present study]. A similar study using *Eptatretus* (Forster *et al.* 1992) also reported the absence of cardioacceleration during mild hypoxia ($P_{W_{O_2}}$=5.3–8.0kPa) despite pronounced effects of catecholamine injections on elevating f_H under normoxic conditions.

Myxine are known to live at great depths, where they may burrow in the mud covering the ocean floor (Foss, 1963). It is conceivable, therefore, that these fish might experience levels of hypoxia even more severe than that used in the initial series on cannulated animals in this study. For this reason, an additional series was performed using anoxic hagfish. Unlike the milder hypoxia, both noradrenaline and adrenaline levels were elevated in the plasma during anoxia, although the former continued to be the predominant circulating catecholamine. The circulating catecholamine levels under conditions of complete anoxia were only slightly greater than those measured during the milder hypoxia. Although these experiments were performed on uncannulated fish, it is unlikely that the method of sampling blood by puncture of the subcutaneous sinus affected the results because there were similar levels of catecholamines in the plasma of cannulated and uncannulated hagfish (compare Table 1 and Fig. 2).

Hagfish were exposed to air for 15min on the assumption that such a treatment would elicit maximal catecholamine release, as previously shown for Atlantic cod (Wahlqvist and Nilsson, 1980; Perry *et al.* 1991) and would thus provide a measure of the *potential* for elevation of plasma catecholamine levels during more physiologically relevant stresses. Interestingly, plasma catecholamine levels were no greater than those levels observed during anoxia. This suggests that maximal catecholamine levels may indeed have been achieved or approached during hypoxia/anoxia.

Although the physiological significance of the elevated circulating catecholamine levels during hypoxia and other acute stresses in hagfish requires further elucidation, it is nevertheless evident from this and earlier studies (Augustinsson *et al.* 1956; Bloom *et al.* 1961, 1962; Johnels and Palmgren, 1960; Ostlund *et al.* 1960; von Euler and Fange, 1961) that the source of these catecholamines is the chromaffin cells within the heart and the great veins. The storage levels measured in the heart agree well with those of previous studies (Bloom *et al.* 1961, 1962; von Euler and Fange, 1961). We are unaware of any earlier studies that have assessed the levels of catecholamines in the posterior cardinal

vein (PCV). The interesting feature of the PCV is the striking predominance of noradrenaline over adrenaline. This may reflect unusually low activities of the enzyme PNMT (phenylethanolamine-*N*-methyl transferase) that catalyses the methylation of noradrenaline to adrenaline (see review by Randall and Perry, 1992). The noradrenaline concentrations in the heart and PCV are comparable to the storage concentrations in the head kidney tissue or PCV of several teleosts, including rainbow trout (head kidney [noradrenaline]=4.5 $\mu g\,g^{-1}$, Nakano and Tomlinson, 1967; PCV [noradrenaline]= 10–15mg g^{-1}, S. G. Reid, unpublished data) and Atlantic cod (PCV [noradrenaline]= 14.3 $\mu g\,g^{-1}$, Abrahamsson and Nilsson, 1976). Indeed, the present study probably underestimates the stored levels in the hagfish because it does not include the contribution of the portal heart.

Clearly, the relatively low (compared with teleosts) circulating catecholamine levels during acute stress in hagfish cannot be explained by inadequate storage levels, but must instead reflect a profound difference in the mechanism of release from the chromaffin cells (see below) or an inordinately greater rate of metabolic clearance/degradation than in other species (see Randall and Perry, 1992). In the experiments in which blood was obtained from subcutaneous sinuses, it is possible that the measured catecholamine levels were low owing to the low turnover time of the blood in these sinuses and the dilution of central blood by the fluid already in the sinuses. The predominance of noradrenaline in the plasma during stress suggests that the PCV may be a more important site of release than the heart or that noradrenaline is released preferentially from *all* storage sites. The differential release of noradrenaline and adrenaline from chromaffin tissue has been demonstrated previously (e.g. Perry *et al.* 1991) and may, in part, reflect distinct populations of noradrenaline- and adrenaline-containing chromaffin cells (Coupland, 1971). Finally, the low levels of circulating catecholamines in *Myxine* may reflect their paracrine action in this species. In other words, catecholamines released from the cardiac chromaffin cells are probably concentrated locally owing to their binding to cardiac adrenoceptors.

In situ *experiments*

The chromaffin cells within the heart of hagfish lack extrinsic innervation and there are no data demonstrating innervation of the PCV. Thus, the predominant mechanism used by fish to initiate release of catecholamines into the blood, the neuronal release of acetylcholine from preganglionic cholinergic nerve fibres, may be unavailable to hagfish. A variety of studies, however, have revealed that the chromaffin tissue of fishes may also release catecholamines in response to local chemical stimuli, including elevated [K^+] (Opdyke *et al.* 1982, 1983), elevated levels of angiotensin II (Opdyke *et al.* 1981), CO_2 (Dashow and Epple, 1985), blood hypoxaemia (Perry *et al.* 1991) and catecholamines themselves (catecholaminotropic effects) (Hathaway *et al.* 1989).

The goal of this study was to develop an *in situ* saline-perfused heart preparation in order to evaluate the potential involvement of localized chemical changes in the initiation of catecholamine release from the chromaffin tissue of hagfish. Owing to our finding of significant catecholamine stores in the PCV and the need to cannulate this vessel, the catecholamines released from this preparation were of course derived both from the PCV

and the heart. The preparation released low levels of catecholamines under non-stimulated conditions whereas application of depolarizing concentrations (40–60mmol l^{-1}) of K^+ caused pronounced non-selective release of adrenaline and noradrenaline. The low basal rate of secretion as well as the stimulation of release following cell membrane depolarization served to validate the preparation for subsequent experiments attempting to reveal the physiological mechanism(s) of catecholamine release.

Plasma [K^+] is elevated in fish after acute stresses, including vigorous exercise (Opdyke *et al.* 1982; Nielsen and Lykkeboe, 1992; Thomas *et al.* 1987), air-exposure (Hyde and Perry, 1987) and hypercapnia (Perry *et al.* 1987). The levels probably also rise during hypoxia as a result of leakage from swollen deoxygenated erythrocytes and from muscle cells owing to the increased physical activity associated with the hypoxia. Opdyke *et al.* (1982) suggested that modulation of plasma K^+ levels might be an important mechanism promoting catecholamine release in primitive vertebrates lacking well-developed sympathetic nervous systems. It does not appear, however, that moderate rises in plasma [K^+] contribute to catecholamine release in hagfish because of the absence of stimulatory effects of [K^+] below 40mmol l^{-1}. This is in contrast to the situation in dogfish, where small exercise-induced increases in plasma [K^+] are thought to supplement the predominant neuronal mechanism of catecholamine release (Opdyke *et al.* 1982).

Perry *et al.* (1991) demonstrated that an *in situ* blood-perfused head kidney preparation of Atlantic cod released adrenaline in response to a lowering of blood O_2 content by approximately 50%. Consequently, it was suggested that local hypoxaemia itself could augment the principal neuronal mechanism of catecholamine release in this species. In the present study, blood O_2 content was reduced by more than 50% during hypoxia (Table 1) so we reasoned that local hypoxaemia might be an important factor causing catecholamine release from the non-innervated hagfish chromaffin tissue. The results clearly indicated, however, that the chromaffin cells of the PCV and heart are unresponsive to hypoxic perfusion fluid and, therefore, a direct influence of hypoxic blood on catecholamine release is unlikely *in vivo*. The reasons for the differences between the two studies are unclear, although it should be pointed out that the previous study of Perry *et al.* (1991) utilized whole blood as the perfusion fluid, whereas the present study used saline.

Although blood pH was not altered during hypoxia (Table 1), it is conceivable that acidosis might accompany more severe stresses, such as anoxia or air-exposure, that elicit catecholamine release. For this reason, we assessed the effects of acidotic perfusion fluid on catecholamine secretion. Lowering the pH of the saline perfusing the heart from approximately 8.1 to approximately 7.0 did not elicit catecholamine release so it is unlikely that acidosis *per se* contributes to the release of catecholamines *in vivo*. Although in teleosts blood acidosis is associated with the mobilization of catecholamines, *in vivo* there is no evidence that acidosis itself is a direct stimulus for catecholamine release (see Randall and Perry, 1992). Indeed, it would appear that acidosis only indirectly induces catecholamine release because of its effect on impairing haemoglobin O_2-binding (Perry *et al.* 1989).

Previous studies on *Myxine* or *Eptatretus* have shown that blood pressure increases during hypoxia (Axelsson *et al.* 1990; Forster *et al.* 1992). Indeed, Axelsson *et al.* (1990) suggested that elevation of venous return pressure might be a factor in the mobilization of catecholamines from the heart. Such a scheme would presumably involve mechanical deformation of the chromaffin cells owing to stretch of the cardiac tissue. It would appear, however, on the basis of the results of the present study that the chromaffin cells of the PCV and heart are insensitive to changes in perfusion pressure, at least within the range 0.6–2.9kPa. The application of the cholinergic receptor agonist carbachol elicited a dose-dependent release of catecholamines, which clearly indicates the existence of cholinergic receptors associated with the chromaffin cells of the PCV and/or heart. Further pharmacological studies to characterize the nature of the cholinergic receptors were not performed, although previous studies on other vertebrate species, including Atlantic cod (Nilsson *et al.* 1976), indicate that they are largely nicotinic; there is, however, evidence in mammals for the additional presence of muscarinic receptors (Chritton *et al.* 1991). The finding of cholinergic release of catecholamines from the heart/PCV of *Myxine* was surprising given the reputed absence of neuronal innervation of the chromaffin cells in this species (see Nilsson, 1983). There are at least two possible explanations for this result. First, it is conceivable that cholinergic (primarily nicotinic) receptors are a fundamental component of the chromaffin cell and that their presence is not dependent on the existence of extrinsic innervation by sympathetic nerve fibres. Second, the focus of previous studies examining the innervation of chromaffin cells in the hagfish was the heart. It is thus possible (although this seems unlikely) that the chromaffin cells associated with the PCV are indeed innervated by sympathetic fibres. It is informative that both adrenaline and noradrenaline were released in response to carbachol (Fig. 5). On the basis of the low storage levels of adrenaline in the PCV, it is likely that the carbachol-induced release of adrenaline originated from the heart, which supports the existence of cardiac chromaffin cell cholinergic receptors. It is unlikely that these receptors play a physiological role in the mobilization of cardiac catecholamines because the naturally occurring cholinergic neurotransmitter acetylcholine does not circulate in the blood.

A ubiquitous feature of the acute stress response in vertebrates is the release of the hypophyseal peptide ACTH (adrenocorticotropic hormone). A recent study has shown that ACTH promotes catecholamine release in a pulmonate snail (Ottaviani *et al.* 1992). Thus, in an exploratory series, pituitary extract of Atlantic cod (it was not possible to obtain hagfish pituitary extract) was injected into the saline perfusing the PCV/heart. The marked stimulation of catecholamine release suggests a possible involvement of one or more pituitary hormones (possibly including ACTH) in the control of catecholamine release in hagfish. Clearly, further experiments are required to elucidate the pituitary factor(s) responsible for causing release and, indeed, we cannot exclude the possibility of endogenous acetylcholine in the pituitary extract as the releasing factor.

In vitro *experiments*

Surprisingly few studies have evaluated the oxygen-binding properties of hagfish blood and the data are sufficiently disparate to permit generalizations on the affinity of

haemoglobin oxygen-binding. A previous study on *Myxine* (Bauer *et al.* 1975) reported a P_{50} value of only 0.56kPa (4.2mmHg), whereas a more recent study on *Eptatretus* (Wells *et al.* 1986) reported a P_{50} value of 1.64kPa (12.3mmHg) at pH7.8 (temperature 16°C). We considered it important to re-evaluate the Hb O_2-binding characteristics of *Myxine* blood, especially considering recent experiments showing that the release of catecholamines in hypoxic teleost fish occurs when Hb O_2-saturation falls to 50–65 % (Thomas *et al.* 1992; Thomas and Perry, 1992; Perry and Reid, 1992). Given the P_{50} value reported for *Myxine* by Bauer *et al.* (1975), blood P_{O_2} would have to decrease to exceptionally low levels (below 1.0kPa) before such levels of Hb O_2-saturation were reached. The P_{50} value of 1.71kPa (12.9mmHg) at pH7.93 (similar to arterial blood pH; see Table 1) was considerably higher than the value reported previously for *Myxine* (Bauer *et al.* 1975) but in agreement with the more recent study on *Eptatretus* (Wells *et al.* 1986). The CO_2 Bohr factor of −1.01 was much higher than previously reported for either *Myxine* (−0.17; Bauer *et al.* 1975) or *Eptatretus* (−0.43; Wells *et al.* 1986).

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