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CONTROL OF CATECHOLAMINE AND SEROTONIN RELEASE FROM THE CHROMAFFIN TISSUE OF THE ATLANTIC HAGFISH

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Summary

An *in situ* saline-perfused systemic heart/posterior cardinal vein preparation of the Atlantic hagfish (*Myxine glutinosa*) was used to assess (1) the ability of the chromaffin tissue to release catecholamines in response to adrenocorticotropic hormone (ACTH; 7.5 i.u. kg^{-1}), serotonin (250 nmol kg^{-1}), carbachol (100 μmol kg^{-1}), [Asn^1-Val^5]angiotensin II (Ang II; 100 nmol kg^{-1}), histamine (0.3–300 μmol l^{-1}) and a high-[K^+] saline (60 mmol l^{-1}), (2) whether serotonin is co-released with the catecholamines of the chromaffin tissues, and (3) the potential modulatory effects of NECA, an adenosine receptor agonist, and DPSPX, an adenosine receptor antagonist, on catecholamine release.

Bolus injections of ACTH, serotonin or carbachol, or perfusion with high-[K^+] saline, all elicited the release of both adrenaline and noradrenaline. Pre-treatment with the serotonergic receptor antagonist methysergide or the cholinergic receptor antagonist hexamethonium abolished the serotonin- and carbachol-mediated catecholamine releases, respectively. Neither receptor antagonist affected the ACTH-mediated catecholamine release. Bolus injections of Ang II or perfusion with a range of histamine concentrations, two potent secretagogues in other vertebrates, did not elicit catecholamine secretion in hagfish.

While injections of Ang II or perfusion with the high-[K^+] saline both elicited the release of serotonin, treatments with ACTH, carbachol or histamine did not. Hence, co-release of catecholamines and serotonin was elicited by non-specific cell membrane depolarization using K^+, but not by the specific secretagogues assessed in this study.

The adenosine receptor agonist NECA and antagonist DPSPX significantly modified the secretory responses elicited by ACTH, serotonin and carbachol. The results suggest that adenosine may inhibit catecholamine release induced by serotonin or carbachol, while stimulating ACTH-induced release.

Although the contribution of the different secretagogues identified in this study has yet to be explored *in vivo*, our results suggest that the control of catecholamine and serotonin release from the aneural chromaffin tissue of the Atlantic hagfish can be achieved through hormonal and/or paracrine means.

Key words: catecholamine, serotonin, hagfish, *Myxine glutinosa*, adrenocorticotropic hormone, adenosine, angiotensin, histamine, carbachol, K^+.

Introduction

In response to an acute physiological challenge, vertebrates release the catecholamine stress hormones adrenaline and noradrenaline (Axelrod and Reisine, 1984; Randall and Perry, 1992) into the circulation. The primary control mechanism of catecholamine release in most vertebrates is through preganglionic sympathetic fibres (Randall and Perry, 1992; Edwards and Jones, 1993). A notable exception to this general pattern is found in hagfish. The principal catecholamine-storing tissue of hagfish, the chromaffin cells of the systemic heart, is not innervated (Green, 1902; Augustinsson *et al.* 1956). So, in principle, the control of catecholamine secretion in these primitive vertebrates should be entirely through non-cholinergic mechanisms.

Hagfish store large quantities of catecholamines in their systemic and portal hearts and posterior cardinal vein (Östlund, 1954; Augustinsson *et al.* 1956; Östlund *et al.* 1960; Euler and Fange, 1961; Perry *et al.* 1993). The morphology and pharmacology of these catecholamine-storing cells are similar to those of the chromaffin cells of the mammalian adrenal medulla (Östlund *et al.* 1960; Bloom *et al.* 1961). The chromaffin cells of hagfish also contain the key enzymes of catecholamine biosynthesis (Jonsson, 1983; Reid *et al.* 1995). Despite these homologies with the chromaffin cells of other vertebrates, recent efforts to investigate possible *in vivo* mechanisms of catecholamine release in hagfish were unable to identify specific secretagogues (Perry *et al.* 1993).

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Previous studies have shown that catecholamines are released during severe hypoxia in hagfish (Perry *et al.* 1993; Bernier *et al.* 1996). However, perfusion of *in situ* preparations with anoxic or acidic saline failed to elicit catecholamine release (Perry *et al.* 1993). Similarly, *in situ* elevation of plasma K^+ concentration within the physiological range also failed to stimulate catecholamine secretion (Perry *et al.* 1993). Although the endogenous catecholamines of the hagfish hearts may be important for the regulation of cardiac function (Bloom *et al.* 1961; Axelsson *et al.* 1990; Johnsson *et al.* 1996) and studies have shown that regulation of the heart is sensitive to the increase in blood pressure observed during hypoxia (Jensen, 1961; Axelsson *et al.* 1990; Forster *et al.* 1992), catecholamine secretion appears to be insensitive to changes in perfusion pressure (Perry *et al.* 1993). Carbachol, a general cholinergic receptor agonist, has been shown to stimulate catecholamine secretion. However, because there is no evidence that the chromaffin cells are innervated, this is not likely to be an important mechanism *in vivo* (Perry *et al.* 1993).

Several other lines of evidence suggest the involvement of hormones and neuromodulators in catecholamine secretion in hagfish. Injections of Atlantic cod (*Gadus morhua*) pituitary extracts elicited a marked release of catecholamines from *in situ* preparations of *Myxine glutinosa* (Perry *et al.* 1993). There is immunohistochemical evidence that the systemic and portal hearts of hagfish contain serotonin (Reid *et al.* 1995), a secretagogue of catecholamine release in rainbow trout (Fritsche *et al.* 1993). Histamine, a potent secretagogue in mammals (Burgoyne, 1991), is also stored in the hagfish hearts (Augustinsson *et al.* 1956). Indirect evidence suggests that angiotensin II (Ang II), a secretagogue throughout vertebrates, may stimulate catecholamine secretion in *Myxine glutinosa* (Carroll and Opdyke, 1982). Finally, *in vivo* treatment of hypoxic Pacific hagfish (*Eptatretus stouti*) with the adenosine receptor antagonist theophylline elicited a marked increase in the circulating concentration of plasma adrenaline (Bernier *et al.* 1996). Thus, the results from these various studies suggest that adrenocorticotropic hormone (ACTH), serotonin, Ang II, histamine and adenosine may all be involved in the control of catecholamine release in hagfish.

The goals of this study, therefore, were (a) to determine whether any of the potential secretagogues listed above do indeed stimulate catecholamine release in *Myxine glutinosa*, (b) to assess whether serotonin is co-released with the catecholamines of the chromaffin tissues, and (c) to investigate the potential modulatory effects of adenosine on catecholamine release.

Materials and methods

Experimental animals

Atlantic hagfish (*Myxine glutinosa* L.) weighing between 26 and 72 g (mean mass 40.6±1.4 g; experimental N=65) were obtained from Huntsman Marine Science Center (St Andrews, New Brunswick, Canada). They were maintained indoors in a large fibreglass tank supplied with recirculated artificial sea water (temperature 5–6 °C). The salinity of the water was kept between 27 and 29 % by partial replacment of the recirculating water as required. The tank was kept covered and the animals were not fed throughout the holding period (from July to November).

In situ *experiments*

To assess the ability of the chromaffin tissue to release catecholamines in response to potential secretagogues, the *in situ* saline-perfused systemic heart/posterior cardinal vein (PCV) preparation of Perry *et al.* (1993) was used with the following modifications. Hagfish were anaesthetized for 30 min in a solution of 2.5 g l^{-1} MS222 (tricaine methanesulphonate) in sea water. The PCV (inflow vessel) and the ventral aorta (outflow vessel) were cannulated with polyethylene (PE 60) tubing stretched to fit the vessels. The cannulated hagfish were immersed in a bath of sea water without anaesthetic and perfused with aerated hagfish saline (pH 8.1; Axelsson *et al.* 1990). The seawater bath and the saline were placed on ice and kept cool (6–8 °C) throughout the perfusions. No movement (muscle contractions) or restoration of consciousness was observed during the perfusions. In all experiments, the *in situ* preparations were perfused for 20 min prior to sample collection. After this stabilization period, two pre-treatment perfusate samples were taken at 1 min intervals. The perfusion saline was then altered in accordance with the treatments described below over a period of 1 min, and seven post-treatment perfusate samples were collected 1, 2, 3, 4, 5, 7.5 and 10 min after the intervention. All samples were collected in pre-weighed 1.5 ml microcentrifuge tubes over a period of 50 s and immediately frozen in liquid N_2. The frozen samples were weighed and stored at –80 °C until analysis of catecholamine content (within 7 days). In series 1, 2 and 3 (see below), the serotonin content of each sample was also determined.

Series 1: the effects of hagfish saline on catecholamine and serotonin release

Hagfish were given a bolus injection (300 µl) of hagfish saline, *via* a valve in the inflow catheter, after the stabilization period, and the response was monitored (N=6). The total perfusion period of each preparation was 30 min.

Series 2: the effects of ACTH, serotonin, Ang II, carbachol and elevated [K^+] on catecholamine and serotonin release

After the 20 min stabilization period and collection of the two pre-treatment samples, a bolus injection (300 µl) of (a) 7.5 i.u. kg^{-1} porcine adrenocorticotropic hormone ($ACTH_{1–39}$, Sigma Chemicals, catalogue no. A-6303), (b) 250 nmol kg^{-1} serotonin oxalate salt (Sigma Chemicals, catalogue no. H-7877), (c) 100 nmol kg^{-1} [Asn^1-Val^5]angiotensin II (Sigma Chemicals, catalogue no. A-6402) or (d) 100 µmol kg^{-1} of the cholinergic receptor agonist carbachol (Research Biochemicals International, catalogue no. C-107) was administered to the preparation *via* a valve in

the inflow catheter and the post-treatment samples were collected. All drugs were prepared daily in hagfish saline. After collecting the post-treatment samples from the first drug injection, the preparation was perfused with aerated hagfish saline for a second stabilization period of 20 min. After this time, two new pre-treatment samples were collected, a second drug was administered and the response was monitored. Using this protocol, the four drugs were randomly tested on each of 10 hagfish. This experimental protocol was chosen to minimize the number of animals used. Once the responses to the four drugs had been assessed, the preparation was perfused for a fifth and final 20 min stabilization period. Once the two pre-treatment samples had been collected, the perfusate reservoir was switched to a high-[K^+] hagfish saline (containing 60 mmol l^{-1} KCl). This protocol is known to provoke a non-specific depolarization of the chromaffin cells and a marked release of catecholamines (Perry *et al.* 1993). Therefore, it was used to assess the viability of the preparations. Overall, the total perfusion period of each preparation was 150 min.

Series 3: the effects of histamine on the release of catecholamines and serotonin

After the 20 min stabilization period and collection of the two pre-treatment samples, the perfusate reservoir was switched to a hagfish saline containing 0.3 μmol l^{-1} histamine and perfusion was continued for 10 min. Once the post-treatment samples had been collected and a new stabilization period had elapsed, this procedure was repeated three more times with saline solutions containing histamine concentrations of 3, 30 and 300 μmol l^{-1} (N=4). Overall, the total perfusion period of each preparation was 120 min. In four other hagfish, after the stabilization period, the preparations were perfused with a hagfish saline containing a single histamine concentration of 300 μmol l^{-1}.

Series 4: the effects of methysergide on catecholamine release

The stimulatory effects of serotonin and ACTH on catecholamine release were assessed using the serotonergic receptor antagonist methysergide (methysergide maleate, RBI catalogue no. M-137) added to the perfusion saline (final concentration 10^{-5} mol l^{-1}). After the 20 min stabilization period and the collection of the pre-treatment samples, a bolus dose of serotonin (250 nmol kg^{-1}) was injected into the infusion cannula. After sampling and re-stabilization, pre-treatment samples were taken and a bolus injection of ACTH (7.5 units kg^{-1}) was administered. Overall, the total perfusion period of each of these preparations was 60 min (N=8).

Series 5: the effects of hexamethonium on catecholamine release

The stimulatory effects of carbachol and ACTH on catecholamine release were assessed using a cholinergic antagonist specific for nicotinic receptors, hexamethonium (hexamethonium dichloride, RBI catalogue no. H-132), added to the perfusion saline (final concentration 10^{-3} mol l^{-1}). The protocol used in this series was identical to that described for series 4. In each preparation, the effects of carbachol (100 μmol kg^{-1}) were assessed first and then, after a second period of stabilization, the effects of ACTH (7.5 units kg^{-1}) were assessed (N=8).

Series 6: assessing the modulatory effects of NECA on catecholamine release

The stimulatory effects of ACTH, serotonin and carbachol on catecholamine release were assessed using the adenosine receptor agonist NECA [5′-(*N*-ethylcarboxamido)adenosine; RBI catalogue no. A-104] added to the perfusion saline (20 mg l^{-1}). NECA is a potent adenosine receptor agonist with nearly equal affinity at A_1 and A_2 receptors (Bruns *et al.* 1986). Using the protocol and the dosages described in series 2, ACTH, serotonin and carbachol were sequentially and randomly tested on each hagfish (N=10). Overall, the total perfusion period of each of these preparations was 90 min.

Series 7: assessing the modulatory effects of DPSPX on catecholamine release

The stimulatory effects of ACTH, serotonin and carbachol on catecholamine release were assessed using the adenosine receptor antagonist DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine; RBI catalogue no. A-022) added to the perfusion saline (20 mg l^{-1}). DPSPX is a potent adenosine receptor antagonist with slight selectivity for A_1 over A_2 receptors (Daly *et al.* 1985). The experimental conditions of this series were similar to those of series 6 (N=15).

Analytical procedures

Perfusate adrenaline and noradrenaline levels were determined on alumina-extracted saline samples (200 μl) using high-performance liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). The HPLC incorporates a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments Corporation, Princeton, NJ, USA). The extracted samples were passed through an Ultratechsphere ODS-C18 5 μm column (HPLC Technology Ltd), using a catecholamine and metanephrine mobile phase (Chromsystems, Munich, Germany). The separated amines were integrated using the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylalamine hydrobromide (DHBA) as an internal standard in all determinations, with a detection limit of 0.1 nmol l^{-1}.

Perfusate serotonin levels were determined on 100 μl samples of non-extracted saline using the same HPLC system and column as above, and with a Cat-A-Phase II mobile phase (Scientific Products and Equipment, Concord, Canada). Calculations of concentrations were based on a linear standard curve, with a detection limit of 1 nmol l^{-1}.

Statistical analyses

Data are presented as mean ± one standard error (S.E.M.). The statistical significance of the observed effects of a given drug injection within a group were tested using one-way repeated-measures analysis of variance (ANOVA). Dunnett's test was used to compare the second pre-treatment sample mean (the pre-treatment sample taken immediately before alterations to the perfusion saline) with values at subsequent and previous times. For a given drug, a paired *t*-test was used to compare the second pre-treatment sample mean (pre-treatment secretion) with the mean of the maximum post-treatment sample values (maximum secretion). The statistical significance of observed differences between the means of several treatments was tested using one-way ANOVA. The significance level for all statistical tests was $P<0.05$.

Results

Series 1: the effects of hagfish saline on catecholamine and serotonin release

Bolus injections of saline did not significantly affect the basal secretion rates of either noradrenaline or adrenaline (Fig. 1). Baseline serotonin secretion was also unaffected by hagfish saline injections (results not shown).

Series 2: the effects of ACTH, serotonin, Ang II, carbachol and elevated [K^+] on catecholamine and serotonin release

Bolus injections of ACTH, serotonin or carbachol resulted in a significant release of both noradrenaline and adrenaline (Figs 1, 2). Although the mean adrenaline secretion rate did not increase significantly in response to serotonin and carbachol injections (Fig. 2F,G), the increase in maximum adrenaline secretion rate was significant (Fig. 1B). This is because the maximum secretion rates account for temporal differences in the release process between preparations, whereas the mean secretion rates do not. Ang II injections did not significantly affect the secretion rate of either noradrenaline or adrenaline (Fig. 1).

Comparison of the results obtained from the random administration of the four potential secretagogues on each *in situ* preparation shows that these results are independent of the order in which these compounds were given. Also, perfusion of the *in situ* systemic heart/PCV preparations with the high-[K^+] saline after application of the four secretagogues stimulated the release of both catecholamines (Figs 1, 2), confirming the viability of the preparations.

Noradrenaline was the predominant catecholamine released in all the treatments, both before and after the injection of drugs or the changes in the [K^+] of the saline (Figs 1, 2). The perfusate noradrenaline/adrenaline secretion rate ratio

Table 1. *The effects of ACTH, serotonin, carbachol, Ang II, histamine and high [K^+] saline on the ratio of noradrenaline to adrenaline secretion rate from the hagfish (*Myxine glutinosa*)* in situ *systemic heart/posterior cardinal vein preparation*

Treatment	Basal NA/AD secretion rate	Maximal NA/AD secretion rate
ACTH (10)	4.21±1.50	1.95±0.74*
Serotonin (10)	5.74±2.52	12.03±5.13*
Carbachol (10)	3.66±1.00	9.07±2.58*
Ang II (10)	4.57±1.84	3.17±1.09
Histamine (8)	8.20±3.06	6.90±1.65
High-[K^+] saline (10)	8.10±2.23	4.85±1.42

Values are the ratio of NA to AD secretion rate levels. The basal values are the samples taken immediately before application of the treatment indicated. The maximal values are the maximum secretion rates recorded after treatment application.

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

* indicates a significant difference from the basal value ($P<0.05$; paired *t*-test).

ACTH, adrenocorticotropic hormone; Ang II, [Asn^1-Val^5]angiotensin II; NA, noradrenaline; AD, adrenaline.

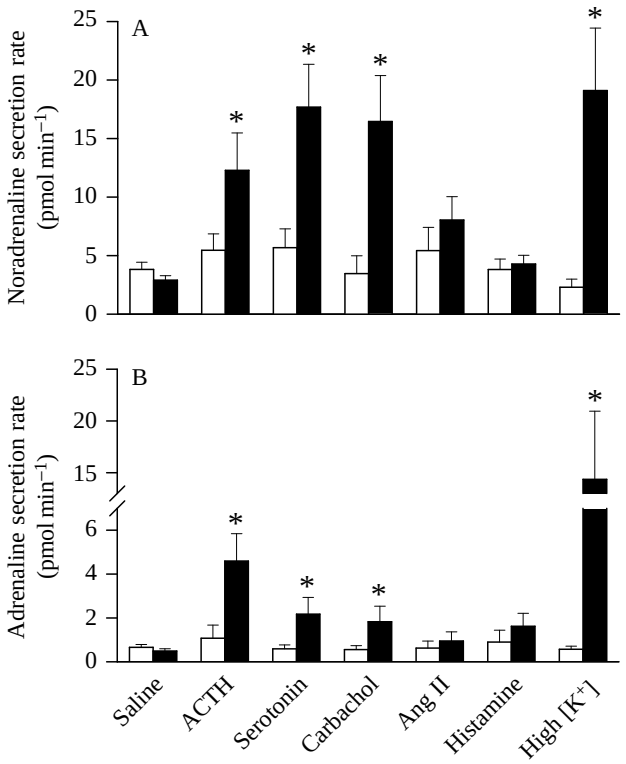

Fig. 1. (A) Noradrenaline and (B) adrenaline secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with physiological saline (control conditions, $N=6$), 7.5 i.u. kg^{-1} porcine adrenocorticotropic hormone (ACTH) ($N=10$), 250 nmol kg^{-1} serotonin ($N=10$), 100 µmol kg^{-1} carbachol ($N=10$) or 100 nmol kg^{-1} [Asn^1-Val^5]angiotensin II (Ang II) ($N=10$) or exposed to saline solutions containing either 300 µmol l^{-1} histamine ($N=8$) or 60 mmol l^{-1} K^+ ($N=10$). The open bars indicate the catecholamine secretion rates prior to the treatments listed above. The filled bars indicate the maximum catecholamine secretion rates in response to those treatments. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment ($P<0.05$). Values are means +1 S.E.M.

decreased significantly from the basal ratio in response to ACTH injections, increased in response to serotonin and carbachol injections, and remained unchanged in response to Ang II and high-[K^+] saline (Table 1).

The high-[K^+] saline perfusion treatment and the addition of Ang II to the preparations both elicited an increase in the secretion rate of serotonin (Fig. 3A,B). ACTH and carbachol treatments had no significant effect on the serotonin secretion rate (Fig. 3C,D). Only 16 % of the control samples had measurable levels of serotonin. All the other control samples were assigned the HPLC detection limit value of 1 nmol l^{-1} in order to facilitate statistical comparisons between pre- and post-treatment samples. Assigning the detection limit value of 1 nmol l^{-1} represents a conservative approach as it will overestimate control values that are below the detection limit.

Series 3: the effects of histamine on catecholamine and serotonin release

Perfusing the *in situ* systemic heart/PCV preparations with hagfish saline containing 0.3–300 µmol l^{-1} histamine did not

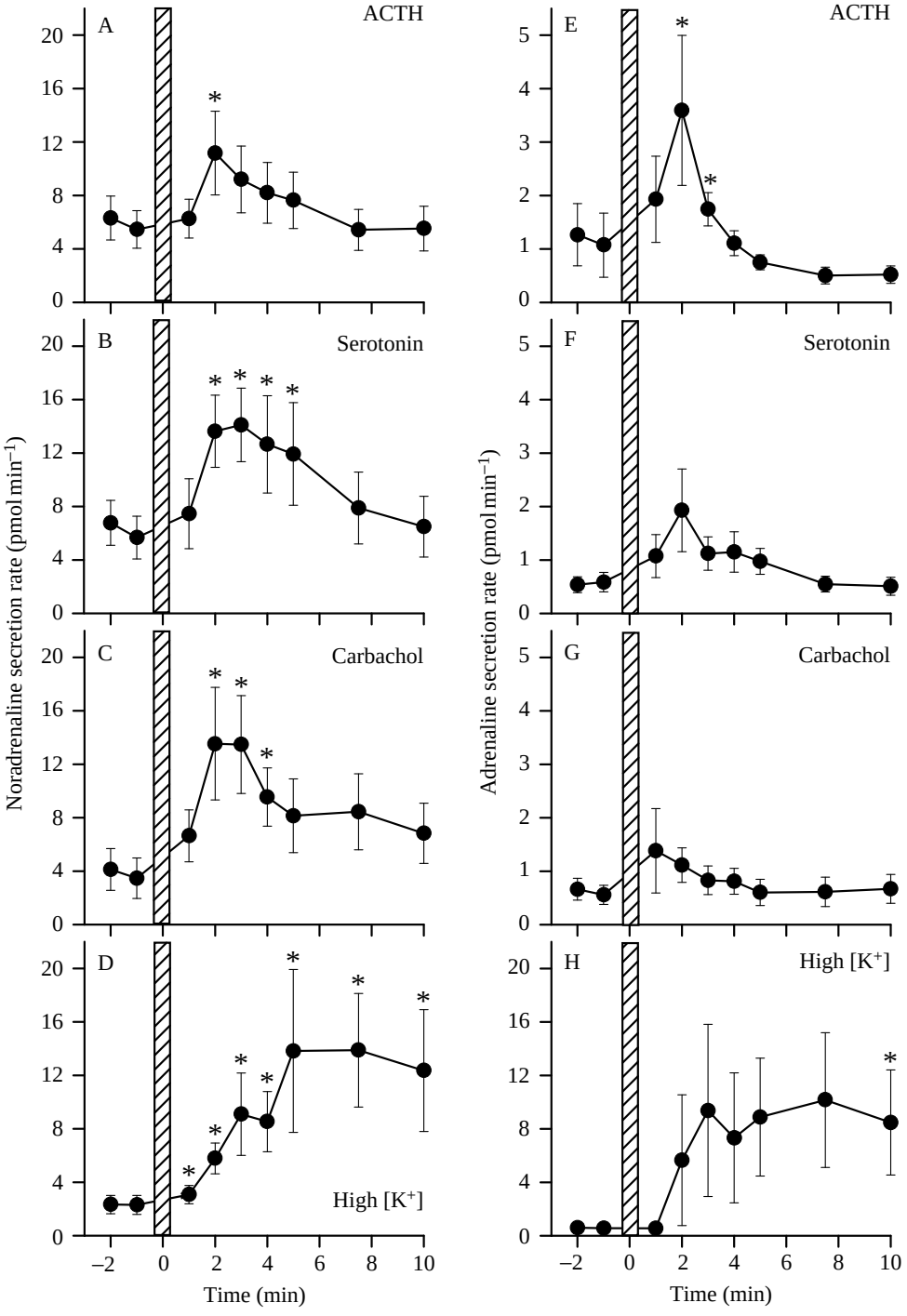

Fig. 2. Noradrenaline (A–D) and adrenaline (E–H) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with ACTH (A,E), serotonin (B,F) or carbachol (C,G) or exposed to a saline solution containing a high [K^+] (D,H). Values to the left of the hatched bar in each plot are the pre-treatment catecholamine secretion rates and values to the right are the secretion rates in response to the treatments listed above. An asterisk denotes a significant difference from the −1 min control value for a given treatment (P<0.05). Values are means ±1 S.E.M. For further details on the treatments and the N values, see the legend of Fig. 1.

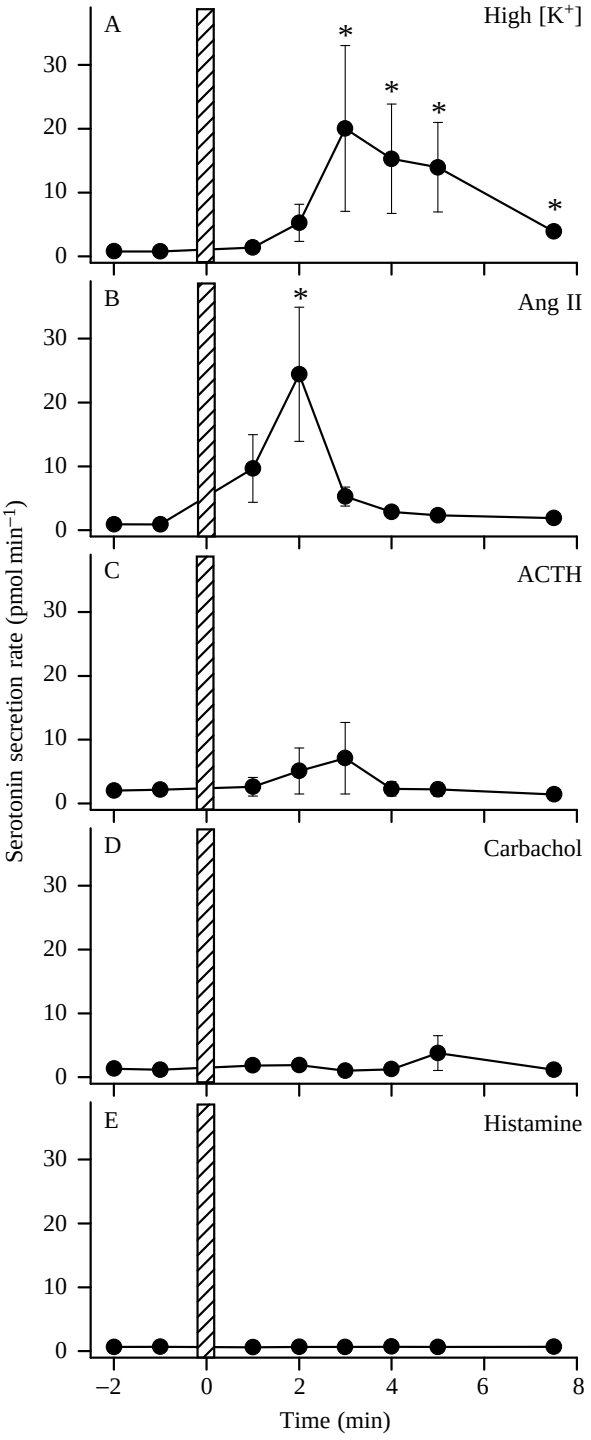

Fig. 3. Perfusate serotonin secretion rate of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with Ang II (N=6) (B), ACTH (N=6) (C) or carbachol (N=6) (D) or exposed to saline solutions containing either a high [K^+] (N=7) (A) or histamine (N=6) (E). Values to the left of the hatched bar in each plot are the pre-treatment serotonin secretion rates and values to the right are the secretion rates in response to the treatments listed above. An asterisk denotes a significant difference from the −1 min control value for a given treatment (P<0.05). Values are means ±1 S.E.M. For further details, see the legend of Fig. 1.

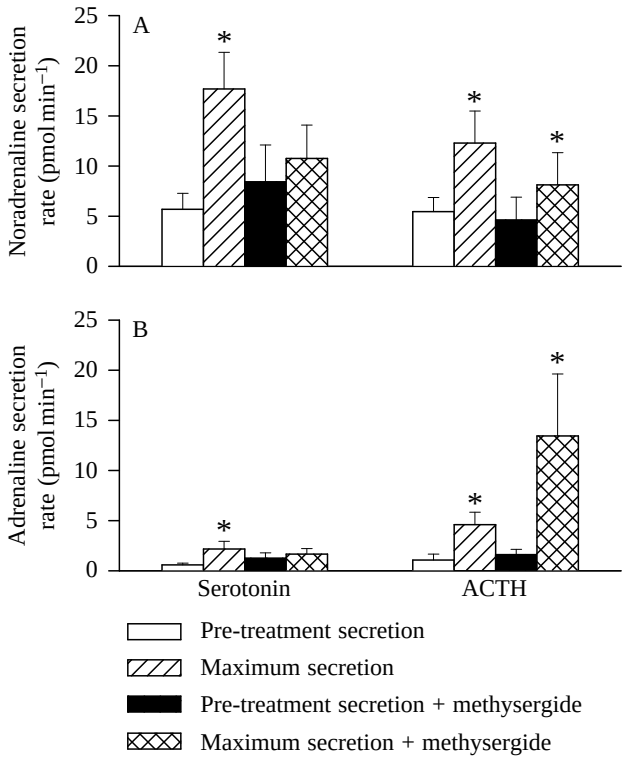

Fig. 4. Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with either 250 nmol kg^{-1} serotonin (N=8) or 7.5 i.u. kg^{-1} porcine adrenocorticotropic hormone (ACTH, N=8). The perfusate consisted of saline only in the groups represented by an open bar or by a diagonally hatched bar. Methysergide (10^{-5} mol l^{-1}), a serotonergic receptor antagonist, was added to the saline in the groups represented by a filled bar or by a cross-hatched bar. The open or filled bars indicate the pre-treatment catecholamine secretion rates prior to the injections listed above. The bars with diagonal or cross hatching indicate the maximum catecholamine secretion rates in response to the injections. An asterisk denotes a significant difference between the control value and the maximum secretion rate for a given treatment (P<0.05). Values are means +1 S.E.M.

stimulate catecholamine secretion above the basal secretion rate (Fig. 1, only data for the 300 µmol l^{-1} treatment are shown). Noradrenaline was the predominant catecholamine released in all the histamine treatments, and the perfusate noradrenaline/adrenaline secretion ratio remained unchanged in response to histamine perfusions (Table 1). Histamine also did not affect the serotonin secretion rate of the preparations (Fig. 3E). In fact, although measurable average amounts of serotonin were detected in the perfusate samples from hagfish injected with all the other potential secretagogues tested, the concentration of serotonin was below the detection limit of HPLC in all the preparations perfused with histamine (Fig. 3).

Series 4: the effects of methysergide on catecholamine release

The presence of methysergide in the perfusion salines prevented the serotonin-induced increase in noradrenaline and

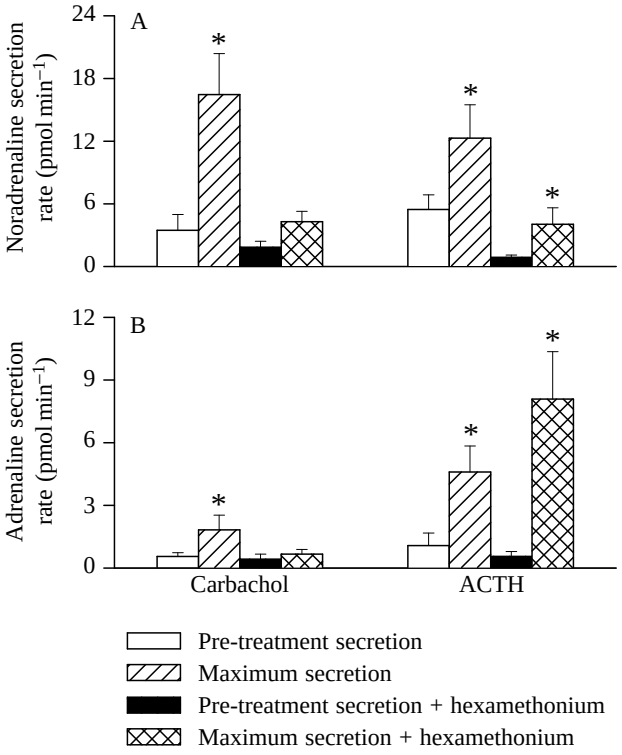

Fig. 5. Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with 100 μmol kg^{-1} carbachol (*N*=8) or 7.5 i.u. kg^{-1} porcine adrenocorticotropic hormone (ACTH, *N*=8). The perfusate consisted of saline only in the groups represented by an open bar or by a diagonally hatched bar. Hexamethonium (10^{-3} mol l^{-1}), a ganglionic receptor blocker, was added to the saline in the groups represented by a filled bar or by a cross-hatched bar. The open or filled bars indicate the pre-treatment catecholamine secretion rates prior to the injections listed above. The bars with diagonal or cross hatching indicate the maximum catecholamine secretion rates in response to the injections. An asterisk denotes a significant difference between the control value and the maximum secretion rate for a given treatment (P<0.05). Values are means +1 S.E.M.

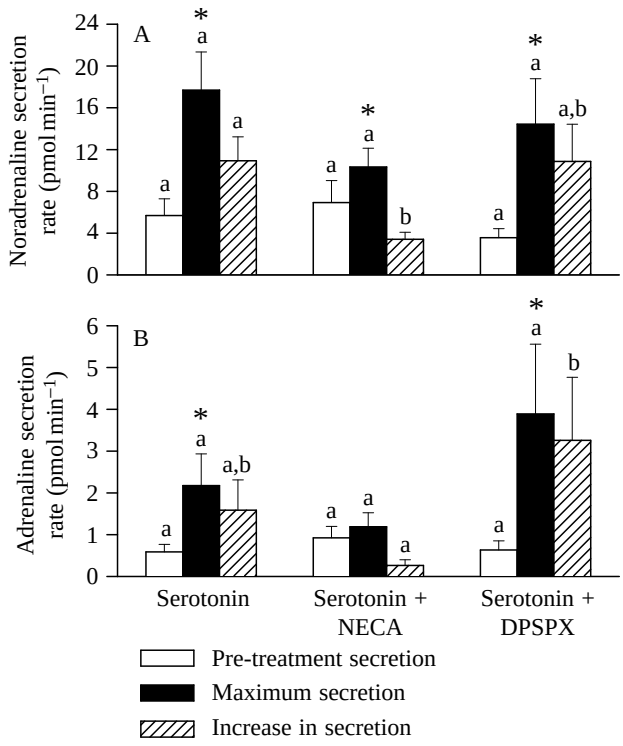

Fig. 6. Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with 250 nmol kg^{-1} serotonin. The preparations were perfused with saline alone (Serotonin, *N*=10), with saline containing the adenosine receptor agonist NECA (Serotonin+NECA, *N*=10) or with saline containing the adenosine receptor antagonist DPSPX (Serotonin+DPSPX, *N*=15). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of serotonin. The filled bars indicate the maximum catecholamine secretion rates in response to the injections. The hatched bars indicate the mean individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other (P<0.05). Values are means +1 S.E.M.

adrenaline secretion rate and had no effect on the ACTH-induced increases in the secretion rates of both catecholamines (Fig. 4A,B).

Series 5: the effects of hexamethonium on catecholamine release

The presence of hexamethonium in the perfusion saline prevented the carbachol-induced increase in catecholamine secretion rate and had no effect on the ACTH-induced increase in catecholamine secretion rate (Fig. 5A,B).

Series 6 and 7: assessing the modulatory effects of NECA and DPSPX on catecholamine release

While the presence of NECA in the perfusion saline of the *in situ* preparations decreased the serotonin- and carbachol-induced stimulation of noradrenaline secretion rate, the presence of DPSPX had no effect (Figs 6A, 7A). NECA also abolished the serotonin- and carbachol-induced release of adrenaline. Relative to the effects of NECA, the addition of DPSPX to the perfusion fluid resulted in a significant increase in the release of adrenaline (Figs 6B, 7B).

The ACTH-induced increase in noradrenaline secretion rate was unchanged by the presence of either the adenosine receptor agonist NECA or the adenosine receptor antagonist DPSPX in the perfusion saline (Fig. 8A). However, the ACTH-induced increase in adrenaline secretion rate was increased by the presence of NECA in the perfusion saline and unchanged by the presence of DPSPX (Fig. 8B).

Overall, irrespective of the secretagogue used to elicit catecholamine secretion from the chromaffin tissue, NECA and DPSPX had greater modulatory effects on the adrenaline secretion rate than on the noradrenaline secretion rate.

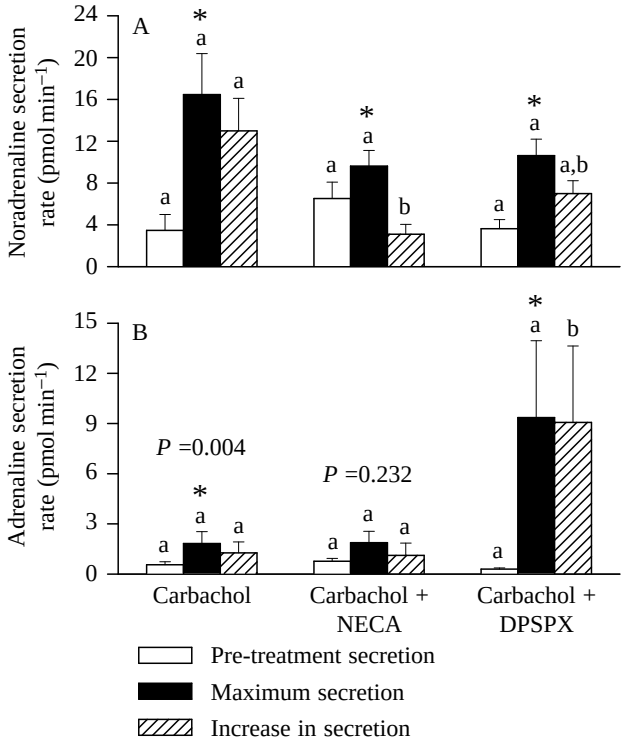

Fig. 7. Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with 100 μmol kg^{-1} carbachol. The preparations were perfused with saline alone (Carbachol, *N*=10), with saline containing the adenosine receptor agonist NECA (Carbachol+NECA, *N*=10) or with saline containing the adenosine receptor antagonist DPSPX (Carbachol+DPSPX, *N*=15). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of carbachol. The filled bars indicate the maximum catecholamine secretion rates in response to the injections. The hatched bars indicate the mean individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. *P* values of the paired *t*-test between the pre-treatment and the maximum secretion rate are shown to emphasize differences between treatments. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other ($P<0.05$). Values are means +1 S.E.M.

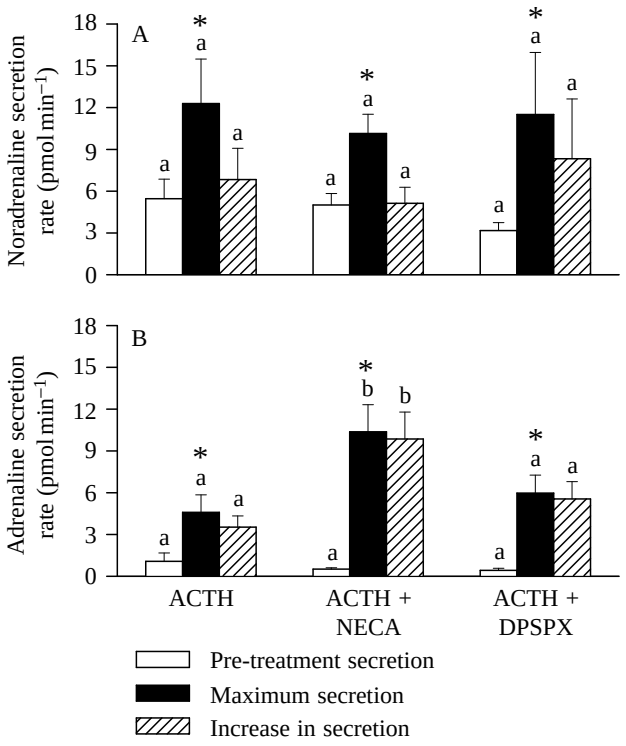

Fig. 8. Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish *Myxine glutinosa* injected with 7.5 i.u. kg^{-1} porcine adrenocorticotropic hormone (ACTH). The preparations were perfused with saline alone (ACTH, *N*=10), with saline containing the adenosine receptor agonist NECA (ACTH+NECA, *N*=10) or with saline containing the adenosine receptor antagonist DPSPX (ACTH+DPSPX, *N*=15). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of ACTH. The filled bars indicate the maximum catecholamine secretion rates in response to the injections. The hatched bars indicate the mean individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other ($P<0.05$). Values are means +1 S.E.M.

Discussion

Secretagogues of catecholamine release

This study presents *in situ* evidence that ACTH, serotonin and carbachol stimulate catecholamine secretion in *Myxine glutinosa*. While ACTH and serotonin are known to stimulate catecholamine secretion in a few vertebrates (Feniuk *et al.* 1980; Chaouloff *et al.* 1992; Fritsche *et al.* 1993; Reid *et al.* 1996), they are the first non-cholinergic secretagogues of catecholamine release to be identified in hagfish. The stimulatory effects of carbachol reported in this study confirm earlier observations (Perry *et al.* 1993). Catecholamine secretion in response to cholinergic receptor agonists (Burgoyne, 1991; Reid and Perry, 1994) and to perfusion with a supra-physiological [K^+] saline (Burgoyne, 1991; Leboulenger *et al.* 1993; Reid and Perry, 1994) are two properties that hagfish chromaffin cells appear to share with the chromaffin systems of other vertebrates. In contrast, the failure of Ang II to elicit catecholamine secretion in the *in situ* saline-perfused systemic heart/PCV preparation appears to be unique among vertebrates (Carroll and Opdyke, 1982). Histamine, a potent secretagogue in higher vertebrates (Burgoyne, 1991), is also without effect on catecholamine release in hagfish.

Adrenocorticotropic hormone

Using a preparation of *Myxine glutinosa* similar to that used in the present study, Perry *et al.* (1993) claimed that ACTH was the hormone most likely to be responsible for

catecholamine release in response to a pituitary extract of *Gadus morhua*. The stimulatory effects of ACTH on catecholamine release reported in this study support this claim.

The possible involvement of ACTH in mediating an acute stress response in hagfish is also supported by the ACTH-like activity of the pituitary gland of *Myxine glutinosa* (Buckingham *et al.* 1985) and by morphological, pharmacological (Fernholm and Olsson, 1969) and immunohistochemical (S. G. Reid, personal communication) evidence for the presence of ACTH-secreting cells in the pituitary gland of *Myxine glutinosa*. However, other immunohistochemical studies, also using antisera against mammalian ACTH in the adenohypophysis of hagfish, have failed to obtain positive results in either *Eptatretus burgeri* (Jirikowski *et al.* 1984) or *Eptatretus stouti* (Nozaki, 1985). Marked differences between the dose–response curves of hagfish pituitary extracts and mammalian ACTH in a cytochemical bioassay (Buckingham *et al.* 1985) and differences in the results obtained in the different immunohistochemical studies discussed above suggest that hagfish ACTH may be structurally distinct from mammalian ACTH.

The only other vertebrate in which ACTH is known to stimulate catecholamine secretion is the rainbow trout *Oncorhynchus mykiss* (Reid *et al.* 1996). As for the results reported in the present study, injections of porcine ACTH elicited a significant secretion of both catecholamines in an *in situ* PCV preparation of the trout (Reid *et al.* 1996). However, while ACTH favours the secretion of noradrenaline over adrenaline in hagfish, the opposite was observed in trout (Reid *et al.* 1996). Moreover, injections of 40 or 200 milliunits per fish of ACTH *in vivo*, as well as extracts of trout pituitary gland given *in situ*, caused an elevation of plasma adrenaline levels, but failed to increase noradrenaline levels in the trout (Reid *et al.* 1996). Although the noradrenaline/adrenaline storage ratio varies considerably between the different sites of catecholamine storage in the hagfish (Augustinsson *et al.* 1956; Östlund *et al.* 1960; Perry *et al.* 1993), and it is not known whether one or all of these sites release catecholamines in response to ACTH stimulation, differences in catecholamine storage between the two species (Perry *et al.* 1993; Reid and Perry, 1994) may explain the differences in the pattern of catecholamine release elicited by ACTH. Among the three secretagogues identified in this study, ACTH elicited the largest secretion of adrenaline and, although carbachol and serotonin injections increased the noradrenaline/adrenaline ratio in the perfusate, ACTH stimulation decreased it.

In hagfish, as in trout (Reid *et al.* 1996), the stimulatory effects of ACTH on catecholamine release are unaffected by pre-treatment with the cholinergic antagonist selective for nicotinic receptors, hexamethonium, or the serotonergic receptor antagonist methysergide. These results suggest that ACTH does not exert its effects by interacting with either serotonergic or cholinergic receptors.

Serotonin

As originally hypothesized by Reid *et al.* (1995), serotonin can directly elicit the release of catecholamines from the chromaffin tissue of hagfish. This serotonin-induced release of adrenaline and noradrenaline is abolished by the serotonergic receptor antagonist methysergide. These results suggest that the stimulatory effects of serotonin are specifically mediated by serotonergic methysergide-sensitive receptors which are located on both adrenaline- and noradrenaline-containing chromaffin cells.

The stimulatory effects of serotonin on catecholamine release have previously been observed in rainbow trout (Fritsche *et al.* 1993). The dosage injected in the *in situ* hagfish preparations, $250\,\text{nmol}\,\text{kg}^{-1}$, also elicits the secretion of catecholamines in rainbow trout under both *in vivo* and *in situ* conditions (Fritsche *et al.* 1993). While serotonin primarily elicits the secretion of noradrenaline in hagfish, the release of adrenaline appears to be favoured in trout (Fritsche *et al.* 1993). As with ACTH-elicited catecholamine secretion (see above), a likely explanation for these differences may be the known differences in catecholamine storage between the two species. For example, while the noradrenaline/adrenaline storage ratios of the PCV and the systemic heart of Atlantic hagfish are approximately 26 and 1, respectively (Perry *et al.* 1993), this ratio is only 0.4 in the PCV of rainbow trout (Reid and Perry, 1994). Results of experiments using methysergide suggest that the serotonergic receptors are present only on the adrenaline-containing chromaffin cells in rainbow trout and that serotonin may also stimulate catecholamine release indirectly *via* the activation of neural pathways (Fritsche *et al.* 1993).

Carbachol

Perry *et al.* (1993) demonstrated that bolus injections of the cholinergic receptor agonist carbachol elicit dose-dependent increases in the release of catecholamines from the *in situ* systemic heart/PCV preparation of *Myxine glutinosa.* Results from the present study confirm the stimulatory effects of carbachol on catecholamine release and show that the response can be abolished by the cholinergic antagonist selective for nicotinic receptors, hexamethonium. Since carbachol is a non-specific cholinergic receptor agonist which stimulates *both* nicotinic and muscarinic receptors, these results suggest that the secretion from adrenaline- and noradrenaline-containing chromaffin cells of *Myxine glutinosa* is controlled exclusively by nicotinic receptors. In general, although the two subtypes of cholinergic receptors can mediate catecholamine secretion from chromaffin cells in vertebrates, their distribution is species-dependent (Parker *et al.* 1993). For example, while the control of catecholamine release in eels (*Anguilla rostrata*; Reid and Perry, 1994) and cod (*Gadus morhua*; Nilsson, 1983), as in hagfish, is mediated only *via* nicotinic receptors, the adrenaline-storing chromaffin cells of rainbow trout also appear to have functional muscarinic receptors (Fritsche *et al.* 1993). Since there is no evidence that the chromaffin cells in hagfish are innervated (Green, 1902; Augustinsson *et al.* 1956),

the presence of nicotinic receptors on these cells may reflect only their common embryological origin as sympathetic neurones (Burgoyne, 1991). However, although the autonomic nervous system is poorly developed in hagfish (Campbell, 1970; Nilsson, 1983), the possibility that the chromaffin tissues of the PCV are innervated cannot be excluded and that the nicotinic receptors of the PCV chromaffin cells may play a physiological role *in vivo*.

Angiotensin

Whereas angiotensins have been shown to stimulate the secretion of catecholamines in animals from elasmobranchs to mammals (Carroll and Opdyke, 1982), [Asn^1-Val^5]Ang II had no effect on the secretion rate of noradrenaline and adrenaline in the *in situ* systemic heart/PCV preparation of *Myxine glutinosa*. The impetus to investigate the effects of Ang II in this study arose from the observation that the pressor response elicited by mammalian angiotensin II (Ang II) could be abolished by adrenergic receptor blockade in hagfish (Carroll and Opdyke, 1982). In view of these results, Carroll and Opdyke (1982) concluded that the pressor response elicited by Ang II in hagfish was mediated entirely by catecholamines. However, although angiotensins elicit a pressor response in *Myxine glutinosa* (Carroll and Opdyke, 1982) and angiotensin-converting-enzyme-like activity has been measured in the liver and plasma of *Eptatretus stouti* (Lipke and Olson, 1988), a complete renin–angiotensin system has not yet been identified in hagfish (Taylor, 1977; Nishimura, 1985). Differences between the results obtained in the present study and that of Carroll and Opdyke (1982) cannot be explained by differences in Ang II dosage. While Carroll and Opdyke (1982) used a dose of 1.91 nmol kg^{-1} of mammalian Ang II, the present study used the much higher dose of 100 nmol kg^{-1} of teleost Ang II ([Asn^1-Val^5]Ang II), the dose that causes maximal catecholamine secretion from the chromaffin tissue of rainbow trout (N. Bernier and S. Perry, unpublished observation). Differences in the amino acid sequence of the Ang II used in the two studies are also unlikely to explain the contradictory results since diverse vertebrate species respond to homologous and nonhomologous angiotensins (Olson, 1992; Silldorff and Stephens, 1992). One possible explanation is that the Ang-II-induced pressor response observed in *Myxine glutinosa* by Carroll and Opdyke (1982) was mediated through the actions of Ang II on the sympathetic nervous system (Wilson, 1984; Reid, 1992). However, the adrenergic autonomic nerve fibres of the hagfish vasculature appear to be poorly developed (Nilsson, 1983). Thus, although Ang II exerts a pressor response, there is no direct evidence for its role in catecholamine release in hagfish.

Histamine

Although histamine elicits a substantial secretory response from the mammalian adrenal medulla and histamine is the most potent non-cholinergic secretagogue in bovine adrenal chromaffin cells (Burgoyne 1991), histamine failed to stimulate catecholamine secretion in the *in situ* systemic heart/PCV preparation of *Myxine glutinosa*. While there is chromatographic evidence that the systemic and portal hearts of *Myxine glutinosa* contain histamine (Augustinsson *et al.* 1956), intravascular injections of histamine yield only weak and inconsistent effects on the systemic and branchial vasculature, and are without effect on the activity of the heart in both *Myxine glutinosa* and *Polistotrema stouti* (Reite, 1969). However, since the effects of histamine on the branchial blood vessels of *Myxine glutinosa* were similar to those obtained with adrenaline and noradrenaline in the same preparation, Reite (1969) suggested that these may be elicited either by direct stimulation of adrenergic receptors or indirectly by the release of endogenously stored catecholamines. The results obtained in the present study do not support the latter hypothesis. Since histamine elicits the release of catecholamines from *in vitro* perfused rat adrenal glands with an EC_{50} of 3 μmol l^{-1} (Borges, 1994), perfusing the *in situ* hagfish preparation with saline solutions containing histamine concentrations ranging from 0.3 to 300 μmol l^{-1} should have been sufficient to elicit potential secretory activity from the chromaffin tissues.

Catecholamine release and noradrenaline/adrenaline secretion ratios

The noradrenaline/adrenaline secretion ratios presented in Table 1 suggest that in hagfish, as in other vertebrates (Accordi, 1991; Chritton *et al.* 1991; Reid and Perry 1994), noradrenaline and adrenaline are stored in different chromaffin cell types. While non-specific cell membrane depolarization with constant high-[K^+] perfusion did not alter the basal noradrenaline/adrenaline secretion ratio, injections of ACTH, serotonin or carbachol all had significant effects. Relative to the control conditions, the significant decrease in the noradrenaline/adrenaline ratio following ACTH injections suggests that ACTH-elicited secretion may arise primarily from adrenaline-storing cells. Similarly, the significant increases in the noradrenaline/adrenaline ratio following serotonin and carbachol injections suggest that catecholamine release elicited by these secretagogues may arise primarily from noradrenaline-storing cells. The high-[K^+] saline was non-selective.

Secretagogues of serotonin release

The presence of serotonin in the systemic heart of *Myxine glutinosa*, as suggested by the immunohistochemical evidence of Reid *et al.* (1995), is confirmed by the perfusion results obtained in the present study. Although serotonin was not detectable in most of the control *in situ* preparations perfused only with hagfish saline, perfusing these same preparations with a high-[K^+] saline elicited a marked release of serotonin. While the effects of high-[K^+] saline on chromaffin cells are non-specific and do not involve the intervention of receptors (Burgoyne, 1991), the serotonin secretion elicited by teleost Ang II ([Asn^1-Val^5]Ang II) in the present study, like the Ang-II-mediated catecholamine secretion in mammals (Marley *et al.* 1989), is probably mediated *via* specific receptors. Since Ang II does not elicit catecholamine secretion in *Myxine*

glutinosa, the effects of Ang II appear to be specific to the control of serotonin release. Moreover, known secretagogues of catecholamine release in hagfish, ACTH and carbachol, had no effect on the serotonin concentration of the perfusate. Hence, although non-specific depolarization of chromaffin cells elicits the release of both catecholamines and serotonin, the secretagogues involved in the control of catecholamine and serotonin secretion may be different. These results also suggest that the serotonin-containing cells of the systemic heart may represent a different population of cells from the catecholamine-containing chromaffin cells.

Immunohistochemical evidence for the presence of serotonin in chromaffin cells has also been reported in several teleost species (Reid *et al.* 1995), in amphibians (Delarue *et al.* 1988) and in mammals (Brownfield *et al.* 1985; Holzwarth and Brownfield, 1985). In rainbow trout, Fritsche *et al.* (1993) showed that serotonin is stored in high concentrations in the anterior region of the PCV within the head kidney. As in hagfish, Reid *et al.* (1995) have suggested that the catecholamine-storing chromaffin cells and the serotonin-storing chromaffin cells of trout may represent different populations. In contrast, the serotonin-containing cells present in the chromaffin tissues of the eel (*Anguilla anguilla*) and the cod (*Gadus morhua*) appear to be analogous to their catecholamine-storing cells (Reid *et al.* 1995). While carbachol induced the release of catecholamines in trout, it failed to elicit the release of the stored serotonin (Fritsche *et al.* 1993). Potential secretagogues of serotonin release in teleosts have yet to be identified.

Since the presence of angiotensins and a complete renin–angiotensin system have yet to be established in hagfish (see above), the functional significance of the Ang-II-elicited serotonin release remains to be determined. Although our results suggest that serotonin ($250\,nmol\,kg^{-1}$ in $300\,\mu l$ of hagfish saline; estimated maximum concentration $33.8\,\mu mol\,l^{-1}$) can elicit catecholamine release, the presence of a lower concentration of serotonin in the perfusate following Ang II injections (maximum secretion $24.7\pm7.9\,nmol\,l^{-1}$) failed to stimulate catecholamine secretion. In addition to a potential paracrine role in the stimulation of catecholamine secretion, the serotonin stored in the systemic heart and PCV of hagfish may have other physiological functions. This is supported by the observations that serotonin has inotropic and chronotropic effects on the systemic heart (Augustinsson *et al.* 1956) and potential vasoactive actions on the gill vasculature of *Myxine glutinosa* (Sundin *et al.* 1994).

Modulatory effects of adenosine on catecholamine release

The adenosine receptor agonist NECA and antagonist DPSPX significantly modified the secretory response elicited by ACTH, serotonin and carbachol. Taken together, the results obtained from the perfusions in the presence of this adenosine receptor agonist or antagonist indicate that adenosine may inhibit the rates of catecholamine secretion induced by either serotonin or carbachol and stimulate those induced by ACTH. Although adenosine and the adenosine analogue PIA (N^6-L-phenylisopropyladenosine) have previously been shown to inhibit the catecholamine secretion elicited by acetylcholine and the nicotinic agonist DMPP (1,1-dimethyl-4-phenylpiperazinium), respectively, in isolated bovine chromaffin cells (Chern *et al.* 1987, 1992), the modulatory effects of NECA and DPSPX on carbachol-, serotonin- and ACTH-induced catecholamine secretion observed in the present study have not been previously reported.

Adenosine, formed from the breakdown of ATP released in parallel with catecholamines (Douglas *et al.* 1965), is thought to have a physiological role in catecholamine secretion from mammalian chromaffin cells by acting as a negative feedback modulator of release from the adrenal medulla (Chern *et al.* 1987, 1992). At the cellular level, adenosine inhibits catecholamine secretion from bovine adrenal medullary cells by reducing agonist-evoked Ca^{2+} fluxes across the plasma membrane (Chern *et al.* 1987). *In vivo*, support for these modulatory attributes of adenosine on catecholamine secretion come from adenosine receptor blockade studies performed on hypotensive rats (Tseng *et al.* 1994), hypoxic rainbow trout and Pacific hagfish (*Eptatretus stouti*; Bernier *et al.* 1996). In *Eptatretus stouti*, whereas exposure to a P_{WO_2} of 10 mmHg (1.3 kPa) for 60 min had no effect on the plasma adrenaline concentration, it increased the latter by 3.8-fold in hypoxic hagfish pre-treated with the adenosine receptor antagonist theophylline (Bernier *et al.* 1996). In the present study, the *in situ* effects of NECA and DPSPX on the pattern of catecholamine secretion induced by serotonin and carbachol agree with the inhibitory properties attributed to adenosine by Chern *et al.* (1987, 1992). In contrast, the interactions between NECA- and ACTH-evoked catecholamine release in *Myxine glutinosa* suggest that adenosine can also play a positive feedback modulatory role. In the presence of forskolin, a drug that enhances the activity of adenylate cyclase and causes a marked rise in intracellular cyclic AMP levels, adenosine, but not NECA, has been shown to enhance catecholamine secretion in isolated bovine chromaffin cells (Chern *et al.* 1988). Hence, although species differences may exist in the signal transduction systems of adenosine receptors, the modulatory effects of adenosine on catecholamine secretion may be either stimulatory or inhibitory and depend on the secretagogue involved.

Overall, NECA and DPSPX had greater modulatory effects on the adrenaline secretion rate than on the noradrenaline secretion rate of the perfused hagfish chromaffin tissue. These observations are consistent with the *in vivo* results obtained in *Eptatretus stouti*, rainbow trout (Bernier *et al.* 1996) and rats (Tseng *et al.* 1994) and, together, they suggest that the modulatory effects of adenosine may be largely aimed at the adrenaline-storing cells.

In conclusion, the *in situ* evidence presented in this study suggests that the chromaffin tissues of *Myxine glutinosa* store both catecholamines and serotonin, and that these hormones may be found in different populations of chromaffin cells. While both serotonin and ACTH stimulate catecholamine

release, Ang II and histamine do not. In contrast, Ang II stimulates serotonin release, whereas ACTH and histamine have no effect. Hence, co-secretion of catecholamines and serotonin is not elicited by the specific secretagogues tested in this study. Our data also reveal that ACTH- and serotonin-elicited catecholamine release can be modulated by adenosine. Although the relative contributions of ACTH, serotonin, Ang II and adenosine have yet to be explored in the overall control of catecholamine and serotonin release *in vivo*, our results suggest that this control can be achieved through hormonal and/or paracrine means.

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