The Journal of Experimental Biology 204, 1247–1255 (2001)
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JEB3148

EVIDENCE FOR A FORM OF ADRENERGIC RESPONSE TO STRESS IN THE MOLLUSC *CRASSOSTREA GIGAS*

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Accepted 10 January; published on WWW 15 March 2001

Summary

Catecholamines and pro-opiomelanocortin (POMC)-derived peptides, some of the central regulators of the stress-response systems of vertebrates, are also present in invertebrates. However, studies are needed to determine how these hormones participate in the organisation of neuroendocrine stress-response axes in invertebrates. Our present work provides evidence for the presence of an adrenergic stress-response system in the oyster *Crassostrea gigas*. Noradrenaline and dopamine are released into the circulation in response to stress. Storage and release of these hormones take place in neurosecretory cells presenting morphological and biochemical similarities with vertebrate chromaffin cells. Both *in vivo* and *in vitro* experiments showed that applications of the neurotransmitters acetylcholine or carbachol caused no significant release of noradrenaline or dopamine. Moreover, the nicotinic antagonists hexamethonium and α-bungarotoxin and the muscarinic antagonist atropine caused no significant inhibition of catecholamine release in stressed oysters. Adrenocorticotropic hormone (ACTH) induced a significant release of noradrenaline, but the release of dopamine in response to ACTH was not significant. These results suggest that, unlike that of vertebrates, the adrenergic stress-response system of oysters is not under the control of acetylcholine and that other factors, such as the neuropeptide ACTH, might control this system.

Key words: catecholamine, noradrenaline, dopamine, acetylcholine, adrenocorticotropic hormone, chromaffin cell, stress, mollusc, *Crassostrea gigas.*

Introduction

The stress response is a series of coordinated physiological reactions increasing the capacity of an organism to maintain homeostasis in the presence of threatening agents. In vertebrates, glucocorticoids together with catecholamines are the major participants in the two main pathways through which the brain regulates the stress response: the hypothalamic/pituitary/steroidogenic cell axis and the hypothalamic/sympathetic nervous system/chromaffin cell axis. In the former pathway, hypothalamic factors, such as corticotropin-releasing hormone (CRH), control hypophysial secretion of pro-opiomelanocortin (POMC)-derived peptides (such as adrenocorticotropic hormone, ACTH) which, in turn, stimulate steroidogenic cells to release glucocorticoids into the blood. In the latter pathway, the hypothalamus controls the release of catecholamines by chromaffin cells through ACTH and cholinergic fibres of the sympathetic nervous system (Chrousos and Gold, 1992; Livett and Marley, 1993; Wendelaar Bonga, 1997). In invertebrates, catecholamines are present in the nerve fibres of a wide range of species (Klemm, 1985; Vaughan, 1988) including coelenterates (Pani and Anctil, 1994), annelids (Anctil et al., 1990), platyhelminths (Gustafsson and Eriksson, 1991), arthropods (Shimizu et al., 1991) and molluscs (Sloley et al., 1990; Takeda, 1992; Pani and Croll, 1995), and POMC-derived peptides have been found in molluscs, arthropods and nematodes (Salzet et al., 1997). Molluscan immunocytes also contain catecholamines, neuropeptides and opioid receptors (Ottaviani et al., 1998; Stefano et al., 1992; Stefano et al., 1993; Stefano et al., 1999; Stefano and Salzet, 1999), and they release biogenic amines when exposed to mammalian CRH or ACTH *in vitro* (Ottaviani et al., 1993).

It appears, therefore, that several messengers, processing enzymes and coordinated hormonal cascades contributing to the stress response exist in both vertebrates and invertebrates. A current theory suggests that these compounds and integrated reactions appeared in ‘ancient’ organisms and have been preserved during the course of evolution (Ottaviani and Fransceschi, 1996). This theory is supported (i) by the finding that ACTH-like material is present in the protozoan *Tetrahymena pyriformis* (LeRoith et al., 1982) and (ii) by the fact that invertebrate biogenic amine receptors and POMC-derived peptides identified to date exhibit high amino acid sequence homology with their mammalian counterparts (Stefano et al., 1993; Salzet et al., 1998). However, further

studies are required to determine how these hormones and receptors contribute *in vivo* to the organisation of a general stress-response system in invertebrates.

This study provides evidence for the existence of an adrenergic response to stress in the oyster *Crassostrea gigas*. By using physiological, pharmacological and histological techniques, the endocrine cells involved in this adrenergic response and molecular processes controlling the stress-induced release of catecholamine have been identified.

Materials and methods

Animals

Oysters *Crassostrea gigas*, Thunberg (60–70 g) were maintained in polyethylene tanks (60–70 oysters per tank) containing 110 l of aerated and continuously flowing (50 $l\,h^{-1}$) natural sea water at 14–15 °C. Animals were left undisturbed for a 10 day acclimation period.

Application of the stressor

Twenty oysters were placed for 15 min in a 20 l plastic container (21 cm in diameter) rotating at 300 revs min^{-1} on a laboratory agitator (model HT, Amilab). The use of a laboratory agitator allows good repeatability of experimental conditions, and preliminary experiments showed that this treatment caused no shell or tissue damage to the animals. After the stress period, the animals were allowed to recover in their original tank. Undisturbed (non-stressed) oysters were used as controls.

Preparation of haemolymph and tissues for catecholamine quantification

Haemolymph (0.5–1 ml) was sampled from the pericardial cavity at 5–10 min intervals before, during and after the application of the stressor. The rapidity of the procedure (1–1.5 min) ensured that the effect of sampling on the stress-induced catecholamine release was kept to a minimum. Samples from 3–5 oysters were pooled. Preliminary experiments showed that variations in catecholamine levels in oysters usually ranged between 8 and 15 % depending on the physiological status and age of the animals. Catecholamine concentration differences between pools ranged between 9 and 11 %. Samples were centrifuged at 600 ***g*** for 10 min to separate the cells from the haemolymph. The supernatants were collected and divided into 1 ml samples to which 50 µl of 10 pg μl^{-1} 3,4-dihydroxybenzylamine (DHBA) was added. Catecholamines were then extracted by absorption on alumina (Goldstein et al., 1981). The extracts were stored at –20 °C and analyzed within 1–2 weeks.

The central nervous system (CNS; including the cerebral, pedal and parietovisceral ganglia), digestive system, mantle, adductor muscle, atria, ventricle and haemocytes (separated from 20 ml of haemolymph) were dissected from stressed and non-stressed oysters. These tissues were weighed and placed in 1 ml of cold 0.1 mol l^{-1} perchloric acid containing 1 ng of DHBA as an internal standard. Samples were homogenized with a cell disruptor (VC 75455, Bioblock Scientific) and centrifuged at 4000 ***g*** for 20 min at 4 °C. The supernatants were collected, stored at –20 °C and analyzed within 1 week.

Catecholamine levels in both the haemolymph and tissues were determined by liquid chromatography with electrochemical detection (Goldstein et al., 1981). The elution peaks from samples were spiked with noradrenaline, adrenaline and dopamine as external standards (Sigma) for confirmation of their identity.

Localization and isolation of catecholamine-containing cells in oyster heart

Hearts were dissected and collected in Hanks' balanced salt solution (HBSS) modified by adjusting to ambient seawater salinity (34 p.p.m.) and by adding 11 mmol l^{-1} D-glucose (MHBSS). To identify the catecholamine-containing cells in the heart, the aldehyde-induced green fluorescence method (Furness et al., 1977) was used. Each heart was cut into four pieces and incubated in a solution of 4 % paraformaldehyde and 0.55 % glutaraldehyde in MHBSS for 24 h at 4 °C. Stained cells were then visualized in cardiac tissues using a BH2 Olympus microscope equipped with a 100 W high-pressure mercury vapour lamp.

To isolate catecholamine-containing cells, hearts were cut into small pieces, transferred to 1 ml of MHBSS containing 0.125 % collagenase (Sigma) and incubated for 15 min with gentle shaking. Heart pieces were then gently triturated and incubated for another 15 min. The supernatant containing the dissociated cells was removed and centrifuged at 100 ***g*** for 10 min at 4 °C. The cell pellet was carefully resuspended in 1 ml of MHBSS and loaded onto a preformed Percoll gradient. Density gradients were prepared by centrifugation at 20 000 ***g*** for 15 min (Beckman L7-55 ultracentrifuge equipped with an SW 41 TI rotor) of a mixture of 9 vols of Percoll (Sigma, density 1.130 g ml^{-1}) with 1 vol of 10-fold-concentrated modified Alsever's solution (Bachère et al., 1988). Cell separation was performed by centrifugation (400 ***g***, 30 min) in swing-out buckets. The catecholamine-containing cell fraction was identified by the chromaffin reaction (Tranzer and Richards, 1976) and the aldehyde-induced green fluorescence method (Furness et al., 1977).

Cell fractions were washed by centrifugation (200 ***g***, 10 min) and resuspended in 0.5 ml of L-15 (Gibco) modified by the addition of NaCl (345.7 mmol l^{-1}), KCl (7.2 mmol l^{-1}), $CaCl_2$ (5.4 mmol l^{-1}), $MgSO_4{\cdot}7H_2O$ (4.1 mmol l^{-1}), $MgCl_2{\cdot}6H_2O$ (19.2 mmol l^{-1}) and 5 % foetal bovine serum (Domart-Coulon et al., 1994). Cells were left to attach onto poly-L-lysine coated glass slides for 60 min and then washed once with MHBSS and incubated in a solution of 4 % paraformaldehyde and 0.55 % glutaraldehyde in MHBSS for 24 h at 4 °C. Characterization of the cells was confirmed by immunostaining of the catecholamine biosynthetic enzymes tyrosine hydroxylase and dopamine β-hydroxylase using the method of Voronezhskaya et al. (Voronezhskaya et al., 1999) with the following modifications: cells were fixed in 3 % paraformaldehyde in MHBSS and then washed twice with PBS (50 mmol l^{-1}

Na_2HPO_4, 140 mmol l^{-1} NaCl, pH 7.4). PBS containing 2 % bovine serum albumin (Sigma) was used to block non-specific binding and to dilute the antibodies. Following an incubation in rabbit anti-tyrosine-hydroxylase (1:1000; Chemicon) and anti-dopamine-β-hydroxylase (1:1000; Chemicon) overnight at 4 °C, cells were washed twice in PBS and incubated for 6 h in Rhodamine-conjugated anti-rabbit IgG (1:100; Chemicon). Omission of the primary antibody served as a methodological control. After 3–4 rinses, all stained cells were mounted in a 3:1 mixture of glycerol and PBS and then viewed under a fluorescence microscope.

In vivo *stimulation of catecholamine release in oysters*

Filtered sea water alone or filtered sea water containing ACTH, acetylcholine or carbachol (Sigma) was injected into the heart *via* a notch made in the valve of the oysters 3–4 days before the experiments. The haemolymph was sampled 5–10 min after the injection and prepared for catecholamine quantification. The effects of the nicotinic acetylcholine receptor antagonists hexamethonium (Sigma) and α-bungarotoxin (Sigma) and of the muscarinic acetylcholine receptor antagonist atropine (Sigma) on stress-induced catecholamine release were determined. Filtered sea water alone or filtered sea water containing hexamethonium, α-bungarotoxin or atropine was injected into the heart. Oysters were subjected, 10 min after the injection, to a 15 min mechanical stress, after which haemolymph was collected for catecholamine quantification.

In vitro *catecholamine secretion by isolated cells*

Isolated catecholamine-containing cells cultured for 60 min in L-15 were incubated for 15 min at 15 °C with or without acetylcholine, carbachol or ACTH in MHBSS supplemented to give 2.5 mmol l^{-1} Ca^{2+} with a corresponding reduction in Na^+ concentration. At the end of the incubation period, the medium was removed, rapidly chilled and centrifuged at 400 ***g*** for 10 min to remove the cells. Both the noradrenaline and dopamine in the medium and that remaining in the cells were then assayed. Amounts of noradrenaline or dopamine released into the medium were expressed as a percentage of noradrenaline or dopamine levels detected in the medium plus that remaining in the cells.

Statistical analyses

All data are presented as means ± S.E.M for at least three experiments. To compare 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 (ANOVA).

Results

Catecholamine concentrations in the haemolymph of stressed oysters

Noradrenaline and dopamine were the only catecholamines released into the oyster haemolymph in response to stress.

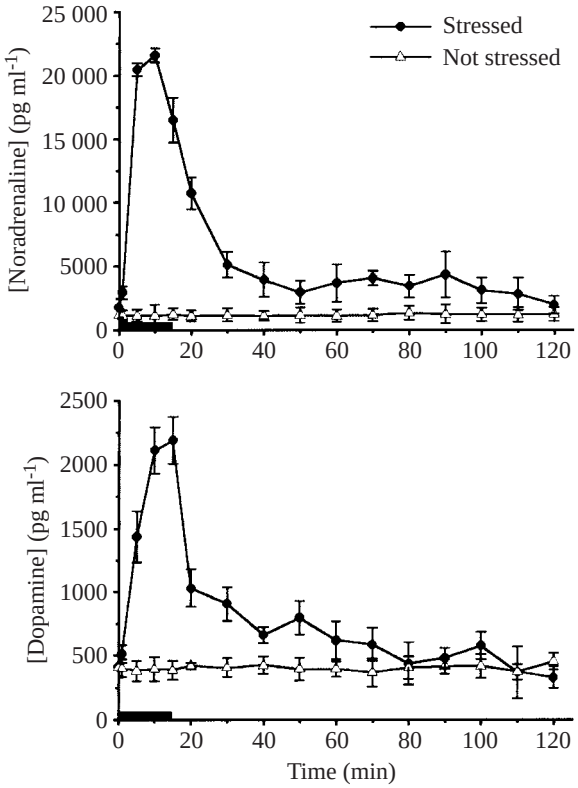

Fig. 1. Effects of a 15 min mechanical stress on circulating levels of noradrenaline and dopamine in the oyster *Crassostrea gigas*. The black bar indicates the duration of the stress. Values are means ± S.E.M. of 3–6 experiments.

Adrenaline was never detected. Noradrenaline was the most abundant catecholamine released; dopamine levels were approximately 10 times lower (Fig. 1). The mean noradrenaline level in resting oysters was 1785.31±774.93 pg ml^{-1} (corresponding to 10.55±4.57 nmol l^{-1}) and the mean dopamine level was 409.45±72.33 pg ml^{-1} (2.66±0.47 nmol l^{-1}). During the 10–15 min exposure to the mechanical stressor, concentrations increased to 21 565.73±550.64 pg ml^{-1} (127.45±3.26 nmol l^{-1}) for noradrenaline (P<0.01) and to 2188.05±182.37 pg ml^{-1} (14.24±1.19 nmol l^{-1}) for dopamine (P<0.01). After the 15 min stress period, catecholamine concentrations decreased rapidly, returning to basal levels 100–120 min after the end of the stimulus.

Catecholamine concentrations in tissues of stressed versus *non-stressed oysters*

Unlike the haemolymph, oyster tissues contained more dopamine than noradrenaline (Fig. 2). Catecholamine concentrations in non-stressed oysters were significantly (P<0.01) higher in the CNS (1183.04±48.92 pg mg^{-1} wet mass for noradrenaline and 1656.33±304.72 pg mg^{-1} wet mass for dopamine), the atria (1189.68±89.26 pg mg^{-1} wet mass for noradrenaline and 936.52±84.88 pg mg^{-1} wet mass for dopamine) and the ventricle (803.29±78.56 pg mg^{-1} wet mass for noradrenaline) than in the other tissues. Following a 15 min

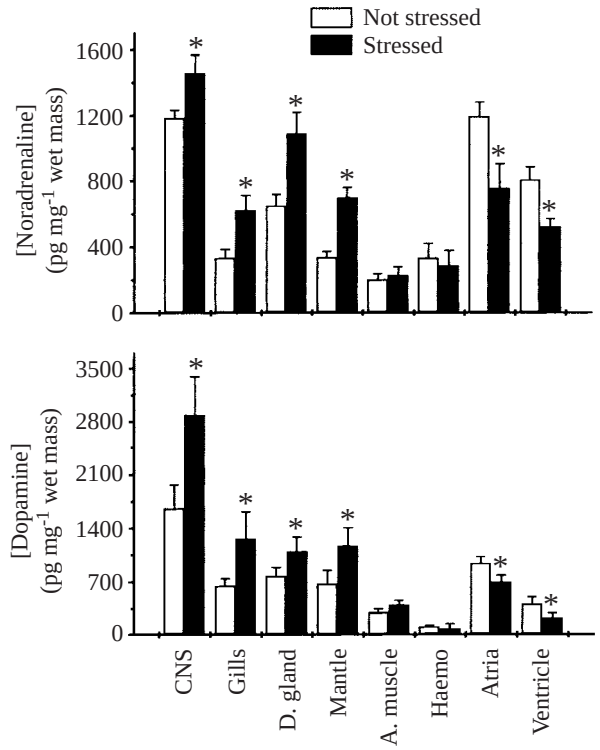

Fig. 2. Concentration of noradrenaline and dopamine in the central nervous system (CNS), gills, digestive gland (D. gland), mantle, adductor muscle (A. muscle), circulating haemocytes (Haemo), atria and ventricle of undisturbed oysters (Not stressed) and of oysters subjected to a 15 min mechanical stress (Stressed). Noradrenaline and dopamine concentrations increased significantly (P<0.01) in all tissues except the adductor muscle and circulating haemocytes, where they remained constant, and in the heart tissues, where they decreased significantly (P<0.01), suggesting that the heart is a site for the release of catecholamines. Values are means + S.E.M. of three experiments. Asterisks indicate significant (P<0.01) stress-induced catecholamine concentration changes.

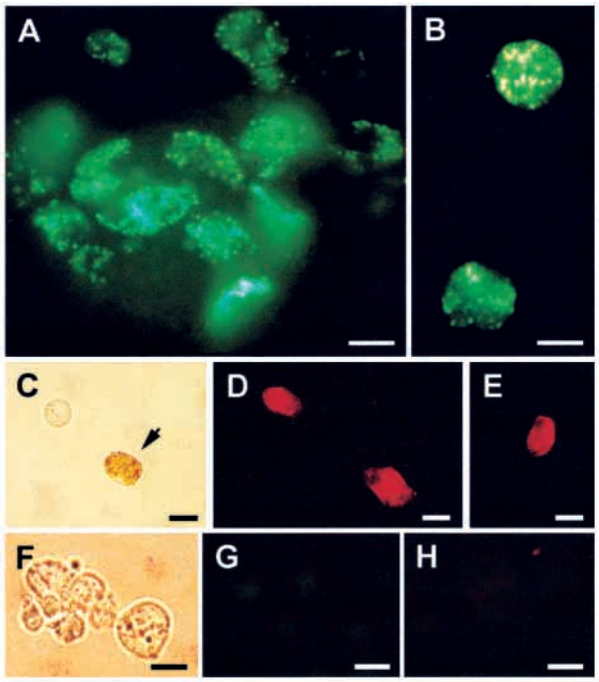

Fig. 3. Evidence for the presence of chromaffin cells in the oyster heart. (A) The cells occur scattered or in small clusters in the heart tissues and, when observed under ultraviolet light, they exhibit the aldehyde-induced blue-green fluorescence characteristic of catecholamines. (B) Isolated cells exhibit the aldehyde-induced blue-green fluorescence and (C) a brownish-yellow appearance in reaction to the chromaffin stain. Immunostaining of the catecholamine biosynthetic enzymes tyrosine hydroxylase (D) and dopamine β-hydroxylase (E) was present throughout the cytoplasm of the isolated cells. (F–H) Negative controls showing non-stained cells observed under visible light (F), the same sample as F observed (same exposure time as A and B) under ultraviolet illumination (G) and a negative control for immunostaining experiments (H) from which primary antibodies were omitted (same exposure time as D and E). Scale bars, 10 μm.

stress, noradrenaline and dopamine levels increased in all tissues except the haemocytes and adductor muscle, where they remained constant, and in the heart, where they decreased significantly (P<0.01).

Catecholamine-containing cells in the heart of oysters

The presence of catecholamine-containing cells in the oyster heart was further investigated using the aldehyde-induced fluorescence method (Furness et al., 1977), which allows the detection of catecholamines in tissues and cells by inducing noradrenaline, adrenaline and dopamine to exhibit a blue-green fluorescence when observed under ultraviolet illumination. Brightly stained cells 12–15 μm in diameter were observed in the heart tissues (Fig. 3A). These cells occurred scattered or in small clusters in both the atria and ventricle and were packed with fluorescent vesicles. No catecholamine-containing fibres were detected in the heart tissue.

Isolated cells also exhibited a blue-green fluorescence (Fig. 3B) indicating that they contained catecholamines. In addition, they exhibited a strong affinity for potassium bichromate (Fig. 3C), indicating their chromaffin nature (Tranzer and Richards, 1976), and were immunoreactive for tyrosine hydroxylase (Fig. 3D) and dopamine β-hydroxylase (Fig. 3E), enzymes involved in the synthesis of catecholamines.

In vivo *stimulation of catecholamine release in oysters*

Cholinergic and non-cholinergic agents were injected into oyster hearts, and their effects on catecholamine secretion were measured. The process itself resulted in very small increases in circulating catecholamine concentrations, as indicated by the controls (Fig. 4). Application of the neurotransmitter acetylcholine at 10^{-4} or 10^{-5} mmol g^{-1} caused no significant release of noradrenaline or dopamine. Similarly, injections of the cholinomimetic carbachol at 10^{-4} or 10^{-5} mmol g^{-1} had no significant effect on catecholamine secretion (Fig. 4A,C). The effects of the nicotinic antagonists hexamethonium and α-

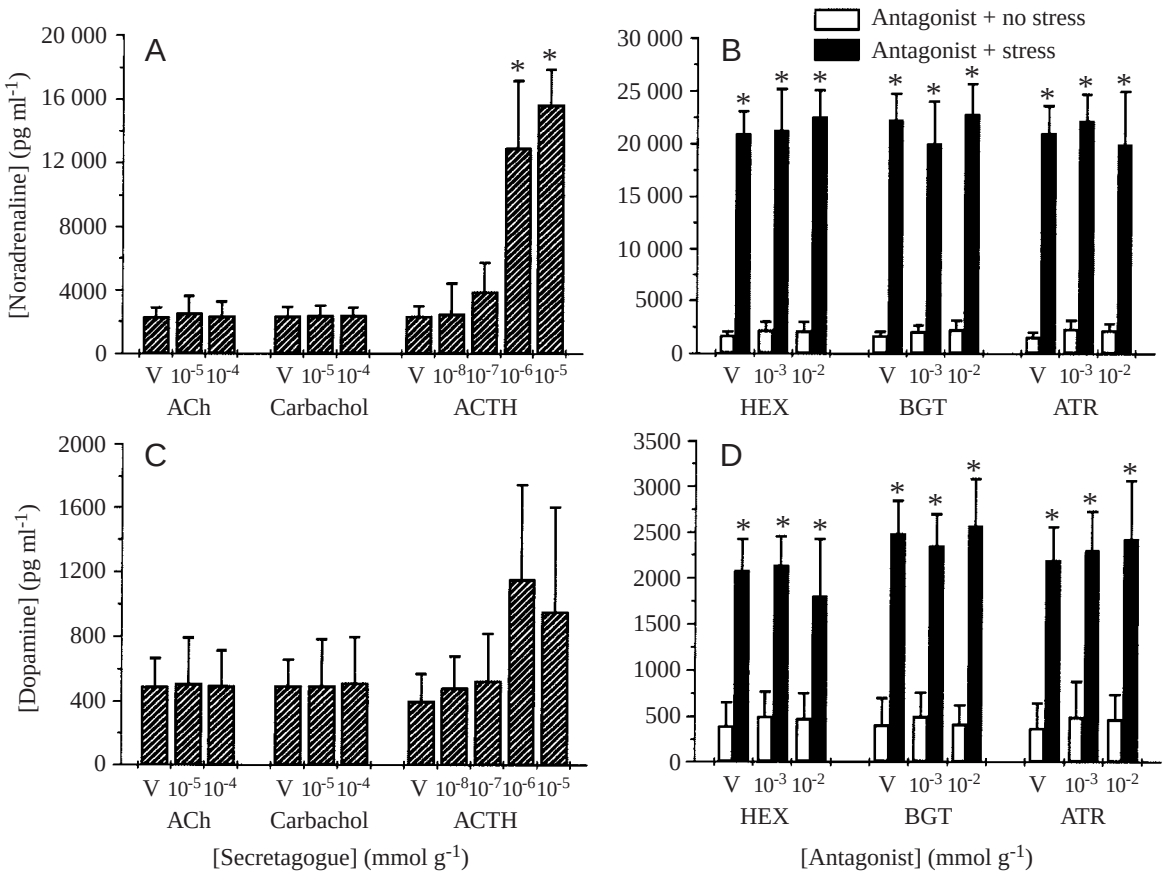

Fig. 4. Effects of secretagogues and acetylcholine-receptor antagonists on catecholamine release *in vivo*. (A,C) Effects of intracardiac administration of acetylcholine (ACh), carbachol and adrenocorticotropic hormone (ACTH) on the concentration of circulating noradrenaline and dopamine in oysters. (B,D) Effects of intracardiac administration of the nicotinic antagonists hexamethonium (HEX) and α-bungarotoxin (BGT) and the muscarinic antagonist atropine (ATR) on the release of noradrenaline and dopamine in stressed oysters. The results showed that the adrenergic response to stress in oysters is not under the control of acetylcholine. In contrast, ACTH induced a significant (P<0.01) release of noradrenaline. V, injection of vehicle alone. Values are means + S.E.M. of three experiments. An asterisk indicates a significant (P<0.01) difference from vehicle-injected oysters (A,C) or unstressed oysters (B,D).

bungarotoxin and of the muscarinic antagonist atropine on stress-induced catecholamine secretion were also tested. None of these compounds showed any intrinsic activity on catecholamine release in non-stressed oysters, and neither the nicotinic antagonists nor the muscarinic antagonist caused any significant inhibition of catecholamine release in stressed oysters when applied at 10^{-3} or 10^{-2} mmol g^{-1}. However, the neuropeptide ACTH induced a significant (P<0.01) release of noradrenaline when applied at 10^{-6} or 10^{-5} mmol g^{-1} (Fig. 4A). The release of dopamine in response to ACTH treatment was not significant (Fig. 4C). As in stressed oysters, noradrenaline was the predominant catecholamine released following the injection of ACTH (15 702.70±2204.62 pg ml^{-1}, or 92.80±13.03 nmol l^{-1} for an ACTH injection of 10^{-5} mmol g^{-1}). Application of ACTH at concentrations of 10^{-7} mmol g^{-1} or below had no significant effect (P>0.01) on levels of noradrenaline.

In vitro *catecholamine secretion by isolated cells*

The purity of the catecholamine-containing cell preparations used in this study was over 70 % as assessed by the aldehyde-induced green fluorescence method (Furness et al., 1977). Basal catecholamine release varied between 0.98 and 1.56 % of the total content (Fig. 5). Incubations either in acetylcholine or in the cholinomimetic carbachol for 15 min at concentrations of 10 and 100 μmol l^{-1} did not cause the catecholamine secretion to increase significantly above basal levels. In contrast, the neuropeptide ACTH, at concentrations of 100 nmol l^{-1}, 1 μmol l^{-1} and 10 μmol l^{-1}, significantly (P<0.01) stimulated the secretion of both noradrenaline and dopamine. The effect of ACTH was concentration-dependent. Noradrenaline was the major catecholamine released following exposure of the cells to ACTH, with secretion ranging from 12.58±1.01 % of the total content at an ACTH concentration of 100 nmol l^{-1} to 26.63±1.16 % of the total content at 10 μmol l^{-1} ACTH (Fig. 5).

Discussion

Previous investigations have demonstrated that the key

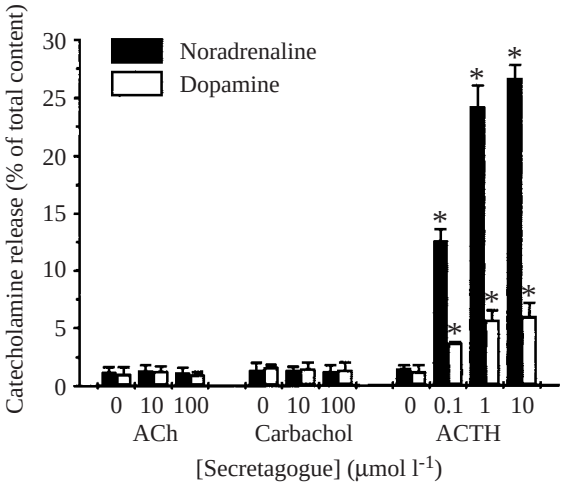

Fig. 5. Noradrenaline and dopamine secretion from isolated oyster chromaffin cells in response to acetylcholine (ACh), carbachol or adrenocorticotropic hormone (ACTH). *In vitro* experiments confirmed that the release of catecholamines from oyster chromaffin cells is not under the control of acetylcholine and that ACTH induces a significant (P<0.01) release of noradrenaline and dopamine. Values are means + S.E.M. of 3–4 experiments performed with different batches of cells. An asterisk indicates a significant (P<0.01) release of noradrenaline or dopamine compared with samples incubated in the absence of drugs. The initial cell content of noradrenaline was 313.6 ng per 10^6 cells, and that of dopamine was 4.7 ng per 10^6 cells.

hormones involved in the stress response in vertebrates (e.g. POMC derivatives and catecholamines) are also present in invertebrates (Ottaviani and Franceschi, 1996; Ottaviani et al., 1998; Stefano and Salzet, 1999; Stefano et al., 1999). However, further studies are needed to determine how these molecules contribute *in vivo* to the organisation of a neuroendocrine stress-response axis in invertebrates. Our results show for the first time that an adrenergic response to stress exists in oysters since noradrenaline and dopamine are released into the haemolymph in response to a mechanical stressor (Fig. 1). The most obvious characteristics of this adrenergic response seem to be (i) high levels of circulating noradrenaline and dopamine and (ii) the absence of adrenaline and the predominance of noradrenaline in the haemolymph. Resting levels of noradrenaline and dopamine measured in oyster haemolymph were 1785.31±774.93 pg ml^{-1} (10.55±4.57 nmol l^{-1}) and 409.45±72.33 pg ml^{-1} (2.66±0.47 nmol l^{-1}), respectively. These levels appear to be higher than those found in the most primitive vertebrates (Hart et al., 1989; Randall and Perry, 1992). Thus, in accordance with previous comparative studies (Hart et al., 1989; Reid et al., 1998), our results suggest that the more primitive the animal the higher the circulating catecholamine levels.

Adrenaline was not detected in the haemolymph of oysters. This result is in accordance with a previous study showing that noradrenaline and dopamine, but not adrenaline, were detectable in *C. gigas* tissues (Osada and Nomura, 1989). Interestingly, small quantities of adrenaline were detected in the haemolymph of the gastropods *Planorbarius corneus* and *Viviparus ater* (Ottaviani and Franceschi, 1996), and we recently detected adrenaline in the haemolymph of the cephalopod *Eledone cirrhosa* (S. K. Malham and A. Lacoste, unpublished results). Thus, it is possible that adrenaline has emerged in molluscs during evolution between bivalves and gastropods from cephalopods. Noradrenaline was the most abundant catecholamine secreted. The predominance of noradrenaline in plasma has been reported only in the most primitive vertebrates such as cyclostomes and elasmobranchs. In teleosts and more evolved vertebrates, adrenaline is the major catecholamine released into the blood (Randall and Perry, 1992; Perry et al., 1993; Wendelaar Bonga, 1997; Reid et al., 1998). Thus, our results converge with others suggesting that the adrenergic stress-response system may have evolved from a noradrenaline-based system requiring high catecholamine concentrations to an adrenaline-based system requiring lower catecholamine concentrations. This trend could be related to the increase in sensitivity of the adrenoceptors (Reid et al., 1998) and the evolutionary up-regulation of the enzymatic processes required for adrenaline biosynthesis (Perry et al., 1993).

In oyster tissues, the most abundant catecholamine was dopamine (Fig. 2). This result is consistent with data collected in other molluscs (Osada and Nomura, 1989; Takeda, 1992; Pani and Croll, 1995). The CNS, ventricle and atria contained higher noradrenaline and dopamine concentrations than the other tissues examined (Fig. 2). Following stress, catecholamine levels remained constant or increased in all tissues, except in the heart, where they decreased, suggesting that this organ is a site for the release of catecholamines into the circulation. However, several studies have shown that the mollusc heart is devoid of catecholaminergic fibres (Carpenter et al., 1971; Smith et al., 1998), a result that contradicts, *a priori*, the presence of catecholamines in this tissue. We did not detect catecholaminergic fibres in the oyster heart, but catecholamine-containing cells were observed in small clusters or scattered singly, both in the ventricle and in the atria (Fig. 3A). These cells are packed with vesicles exhibiting the aldehyde-induced blue-green fluorescence (Furness et al., 1977) characteristic of catecholamines (Fig. 3B) and have a brownish-yellow appearance in the presence of chromium salts (Fig. 3C), indicating their chromaffin nature (Tranzer and Richards, 1976). In addition, they are immunoreactive for the catecholamine biosynthetic enzymes tyrosine hydroxylase and dopamine β-hydroxylase (Fig. 3D,E) and they contain both noradrenaline and dopamine (Fig. 5). As such, they resemble vertebrate chromaffin cells.

The possibility that analogous mechanisms have evolved in parallel cannot be excluded, but our results and other comparative studies on fish support the conclusion that, since a systemic heart first appeared in bivalves (Eble, 1996), this organ exerted an important role in the secretion of catecholamines as soon as it emerged in the animal kingdom and until elasmobranchs and teleosts appeared. Indeed, in

cyclostomes, the chromaffin cells are also located into the systemic heart. Only in higher vertebrates did the chromaffin tissue evolve into the adrenal gland (Reid et al., 1998). It is noteworthy that, humans, like other mammals, are known to possess extra-adrenal chromaffin tissues, and the heart contains chromaffin cells that exert a direct paracrine action (Almeida et al., 1985; Gobbi et al., 1991).

The primary mechanism leading to the secretion of catecholamines from the chromaffin tissue in elasmobranchs and higher vertebrates is the stimulation of the chromaffin cells by preganglionic sympathetic fibres *via* the neurotransmitter acetylcholine (Reid et al., 1998). In the majority of vertebrate species, the nicotinic receptor is the predominant cholinoceptor involved in the stimulation of chromaffin cells by acetylcholine (Reid et al., 1998). As a consequence, the secretion of catecholamine by chromaffin cells can be stimulated by acetylcholine or the mixed nicotinic/muscarinic agonist carbachol (Randall and Perry, 1992; Reid et al., 1998) and can be inhibited by the nicotinic antagonists hexamethonium and α-bungarotoxin (Reid et al., 1998; Lopez et al., 1998). In molluscs, carbachol is known to mimic the effects of acetylcholine, and hexamethonium, α-bungarotoxin and atropine antagonize the effects of this neurotransmitter in nervous fibres and cardiac muscle cells (Rosza, 1984; Gebauer and Versen, 1998). Although cholinergic fibres are thought to be present in the heart of molluscs (Hill and Kuwasawa, 1990), our results show that, in oysters, neither intracardiac injections of acetylcholine (Fig. 4) nor incubation of chromaffin cells in acetylcholine (Fig. 5) stimulated the release of catecholamine. Carbachol, which is more resistant than acetylcholine to degradation by endogenous cholinesterases, also had no significant effect. Furthermore, neither the nicotinic antagonists hexamethonium and α-bungarotoxin nor the muscarinic antagonist atropine inhibited the increase in levels of circulating catecholamines following stress.

Interestingly, in the Atlantic hagfish *Myxine glutinosa*, acetylcholine induces the release of catecholamines from chromaffin cells present in perfused heart preparations, but this neurotransmitter is not thought to regulate the secretion of catecholamines *in vivo*, given the reputed absence of neuronal innervation of its chromaffin tissue (Perry et al., 1993; Reid et al., 1998). In this primitive fish, the release of catecholamines is thought to be under the control of non-cholinergic pituitary factors (Perry et al., 1993; Bernier and Perry, 1996; Reid et al., 1996). Indeed, ACTH stimulates the release of catecholamines from hagfish heart preparations (Bernier and Perry, 1996). The presence of POMC derivates and their receptors has been demonstrated in various invertebrates including molluscs (Stefano and Salzet, 1999), and ACTH has been shown to circulate at concentrations of $30\,nmol\,l^{-1}$ in mussel haemolymph (Stefano et al., 1999) and at concentrations of $6–35\,nmol\,l^{-1}$ in the haemolymph of the rhynchobdellid leech *Theromyzon tessulatum* (Salzet et al., 1997). Moreover, this hormone is thought to be involved in the neuroendocrine response to stress in bivalves and gastropods (Ottaviani et al., 1991; Ottaviani et al., 1998).

As a consequence, we tested the hypothesis that ACTH plays a role in the control of catecholamine secretion in oysters. Our results show that, in *C. gigas*, the release of catecholamine can be induced *in vivo* by intracardiac administration of ACTH (Fig. 4) and *in vitro* by the incubation of chromaffin cells in the presence of $\geqslant 100\,nmol\,l^{-1}$ ACTH (Fig. 5). Although $30\,nmol\,l^{-1}$ appears to be the normal ACTH concentration in mussel haemolymph (Stefano et al., 1999), ACTH-induced catecholamine release was not significant at doses below $100\,nmol\,l^{-1}$ ACTH. This may be due to the reduced sensitivity of chromaffin cells *in vitro*. Alternatively, ACTH concentrations may increase above $30\,nmol\,l^{-1}$ in stressed oysters, leading to the release of catecholamine by chromaffin cells. Although data are lacking concerning stress-induced ACTH concentration changes in mollusc haemolymph, this second hypothesis would be consistent with previous studies suggesting that ACTH may be released during stress in molluscs (Ottaviani and Franceschi, 1996).

Taken together, our results show that the stress-induced secretion of catecholamines in oysters is not under the control of cholinergic factors. Other hormonal messengers, such as the neuropeptide ACTH, appear to control the release of catecholaminergic substances in this mollusc. The involvement of acetylcholine in the release of catecholamines from chromaffin cells seems to have appeared in more evolved animals, since only in elasmobranchs and teleosts did it become a predominant pathway leading to the secretion of catecholamines by the chromaffin tissue (Reid et al., 1998). It is possible that the emergence of nervous control of catecholamine secretion was induced by a necessity to develop distinct stress-response systems, one involving factors such as ACTH and ACTH-activated effectors such as cortisol, and the other involving the sympathetic nervous system and catecholamines. Presumably, the advantage of dividing one stress-response system into two separate axes would be to enable the animal to elicit different neuroendocrine responses depending on the nature of the stress encountered. This hypothesis is in keeping with other studies disputing the validity of Selye's concept of nonspecificity of the neuroendocrine response to stress (Selye, 1998). It is also strongly supported by a recent study (Pacak et al., 1998) that emphasized the stressor specificity of neuroendocrine responses in rats by showing that adrenocorticotropic and adrenergic responses to stress are not systematically related.

This work was supported by grants from the North Brittany research program GIGASMOR and from the Conseil Régional de Bretagne. We thank Drs Serge Thomas and Laurent Meijer for critical reviews of the manuscript.

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