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Stress-Induced Catecholamine Changes in the Hemolymph of the Oyster *Crassostrea gigas*

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The stress response is a series of coordinated physiological reactions increasing an organism's capacity to maintain homeostasis in the presence of threatening agents. This fundamental process is known to involve hormonal signaling to rapidly modulate key physiological functions in vertebrates, but data are lacking concerning neuroendocrine responses to stress in invertebrates. The present study examined circulating catecholamine (CA) responses to stress in oysters. Mechanical disturbances (consisting of shaking the animals) and temperature or salinity variations were applied to the animals because these three types of stressors are commonly encountered by oysters in aquaculture or in their natural habitat. Results show that both circulating noradrenaline (NA) and dopamine (DA) concentrations increased in response to stress. The catecholaminergic response to acute mechanical stressors was rapid (less than 5 min), transient (a return to basal CA levels was observed after 60–90 min), and reflected both the intensity and duration of the perturbation. In contrast, responses to temperature and salinity variations were long lasting (up to 72 h). CA concentrations varied from 1.61 ± 0.30 ng NA/ml and 0.41 ± 0.05 ng DA/ml to maximal values of 22.07 ± 0.97 ng NA/ml and 2.24 ± 0.19 ng DA/ml. Such CA concentrations are known to induce physiological responses in bivalves, suggesting that stress-induced NA and DA changes exert a regulatory function in oysters.

Key Words: Crassostrea gigas; mollusc; catecholamines; noradrenaline; dopamine; stress.

INTRODUCTION

Virtually no habitat on earth is static. As a consequence, the homeostatic equilibrium of any living organism is constantly challenged or threatened by diverse disturbing forces known as stressors. To maintain homeostasis, stressed animals elicit a coordinated set of behavioral and physiological responses, also termed "stress response," to adapt and overcome threatening situations (Wingfield and Ramenofsky, 1999).

Once detected by sensory organs, stress results primarily in the stimulation of two major neuroendocrine axes in vertebrates: the hypothalamic–pituitary–steroidogenic cell axis and the hypothalamic–sympathetic nervous system–chromaffin cell axis (Chrousos, 1992; Wendelaar Bonga, 1997). The former pathway involves neuropeptides and glucocorticoids as major messengers, whereas the latter pathway involves catecholamines (CA). In turn, glucocorticoids and CA induce secondary responses, including increased oxygen uptake, mobilization of energy substrates, and reallocation of energy away from nonimmediately required physiological processes, such as growth, reproduction, and certain immune functions (Wendelaar Bonga, 1997).

Little information is available concerning the presence of glucocorticoids in invertebrates (Ottaviani *et al.*, 1998) but CA are present in many phyla (Klemm, 1985; Vaughan, 1988), including coelenterates (Pani

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and Anctil, 1994), annelids (Anctil *et al.*, 1990), platyhelminthes (Gustafsson and Eriksson, 1991), and arthropods (Bakary *et al.*, 1988; Shimizu *et al.*, 1991). CA and several other biogenic amines have also been detected in various mollusc tissues (Sloley *et al.*, 1990; Takeda, 1992; Pani and Croll, 1995, 1998; Rosza, 1984; Vornezhskaya *et al.*, 1999) and in gastropod and bivalve hemolymph (Manger *et al.*, 1996; Santhanagopalan and Yoshino, 2000; Pani and Croll, 2000). In molluscs, CA play essential roles in several physiological processes, including feeding (Teyke *et al.*, 1993), locomotion (Sakharov and Salánski, 1982), respiration (Syed and Winlow, 1991), reproduction (Martínez and Rivera, 1994), and larval settling and metamorphosis in free-living marine species (Pires *et al.*, 1997). Little is known, however, about how stress influences CA secretion and metabolism in these animals.

The present study investigates the effects of mechanical stressors and temperature variations or salinity decrease on CA concentrations in the hemolymph of the bivalve *Crassostrea gigas.*

MATERIALS AND METHODS

Animals

C. gigas oysters (60–70 g) were maintained in polyethylene tanks (60 to 70 oysters per tank) containing 110 l of aerated and continously flowing (50 liter/h) natural seawater at 14°–15°C. Animals were left undisturbed for a 10-day acclimation period before experiments. Oysters were submitted to various types of stressors, including shaking, temperature increase (from 15° to 28°C) or temperature decrease (from 15° to 5°C), and salinity decrease (from 34 to 24 ppm). For shaking, oysters were placed in a 20-liter plastic container (21 cm in diameter) rotating on an HT laboratory agitator (Amilabo). This procedure was chosen because it reproduces situations encountered by the animals during sorting or grading processes in oyster farms. The use of a laboratory agitator allows good repetition of experimental conditions and previous experiments showed that this treatment caused neither damage to oyster tissues nor breaks in the shells. After the stress period, the animals were allowed to recover in their original tank. Oysters, initially acclimated to 15°C and 34 ppm seawater, were carefully immersed in either 28° or 5°C seawater or in seawater adjusted to 24 ppm by addition of distilled water. Undisturbed (nonstressed) oysters were used as controls.

Preparation of Hemolymph and CA Quantification

For all experiments, each oyster was taken from its tank immediately prior to sampling. The adductor muscle was rapidly severed, the right valve was removed, hemolymph (0.5–1 ml) was sampled from the pericardial cavity using 2-ml syringes and 26-gauge × 1/2-in. needles, and samples were put on ice. The rapidity of the procedure (1–1.5 min per sample) ensured that the effect of sampling on the stress-induced CA release was kept minimal. Samples from three to five oysters were pooled, centrifuged at 600*g* for 10 min at 4°C to remove the cells from the hemolymph, and immediately processed for CA extraction. Cell-free supernatants were collected and divided into 1-ml aliquots to which 50 μl of 10 pg/μl 3,4-dihydroxybenzylamine (DHBA) was added. CA were then extracted by absorption on alumina (Goldstein *et al.*, 1981). Extracts were stored at −20°C and analyzed within 1–2 weeks. CA levels were determined by liquid chromatography with electrochemical detection (HPLC–ED). The HPLC system consisted of a Waters 515 HPLC Pump equipped with an injection valve containing a 100-μl sample loop. CA detection was accomplished by means of a Model 105 electrochemical detector (Precision Instruments, Marseille, France). The electrochemical potential was adjusted at 0.6 V against a silver/silver chloride reference electrode and a glassy carbon working electrode. Analyses were performed in isocratic mode at a rate of 1 ml/min. The samples were analyzed using a Merck mobile phase and column specifically designed for the determination of noradrenaline (NA), adrenaline (A), and dopamine (DA). The electrochemical signal from the detector was transmitted to a computerized data acquisition system (Borwin 1.5, JMBS). CA concentrations were calculated by the internal standard method using peak areas and taking into account the recovery rate (70–90% referred to an external standard solution) determined for each sample. The elution peaks from samples were spiked with NA, A, and DA external standards (Merck) for confirmation of their identity.

Statistical Analysis

All data are presented as means and standard errors of at least three experiments. 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.

RESULTS

Chromatograms presented in Fig. 1 show that NA and DA could be detected and quantified by HPLC–ED in *C. gigas* cell-free hemolymph. Mixing oyster hormonal extracts with an external standard solution containing NA, A, DHBA, and DA confirmed that the peaks obtained from oyster hemolymph were NA and DA (Fig. 1B). Adrenaline was not detected in oyster hemolymph. Both NA and DA peaks increased in oysters submitted to shaking for 1 min (Fig. 1C). NA and DA levels in resting oyster cell-free hemolymph were 1.61 ± 0.30 and 0.41 ± 0.05 ng/ml, respectively (Fig. 2). Concentrations increased to 22.07 ± 0.97 ng/ml for NA ($P < 0.01$) and 2.24 ± 0.19 ng/ml for DA ($P < 0.01$) upon exposure to a mechanical stressor by shaking the animals for 15 min on a laboratory agitator rotating at 300 rpm. When oysters were submitted to shaking at 100 rpm, circulating NA and DA concentrations reached 6.58 ± 0.56 and 1.21 ± 0.11 ng/ml, respectively. After the stress, CA concentrations decreased rapidly, returning to basal levels 100 to 120 min after the end of the stimulus. Results in Fig. 3 show that a 1-min shaking stress was sufficient to elicit a fivefold increase in circulating NA and a twofold increase in circulating DA. The maximal NA and DA responses in oysters submitted to shaking for 60 min were not significantly higher than those observed in response to shaking for 15 min; however, NA and DA concentrations remained, respectively, ≥10 times and ≥2 times higher than initial resting levels until the end of the 60-min stress period. In oysters that were subjected to repeated 15-min shaking periods (Fig. 4), poststress CA levels remained three- to fivefold higher than basal levels ($P < 0.01$) and the amplitude of the hormonal response decreased significantly ($P < 0.01$) after the second repetition. The amplitudes of NA and DA responses to the sixth stress repetition were, respectively, ≤67% ($P < 0.01$) and ≤25% ($P < 0.01$) of the amplitude of initial responses.

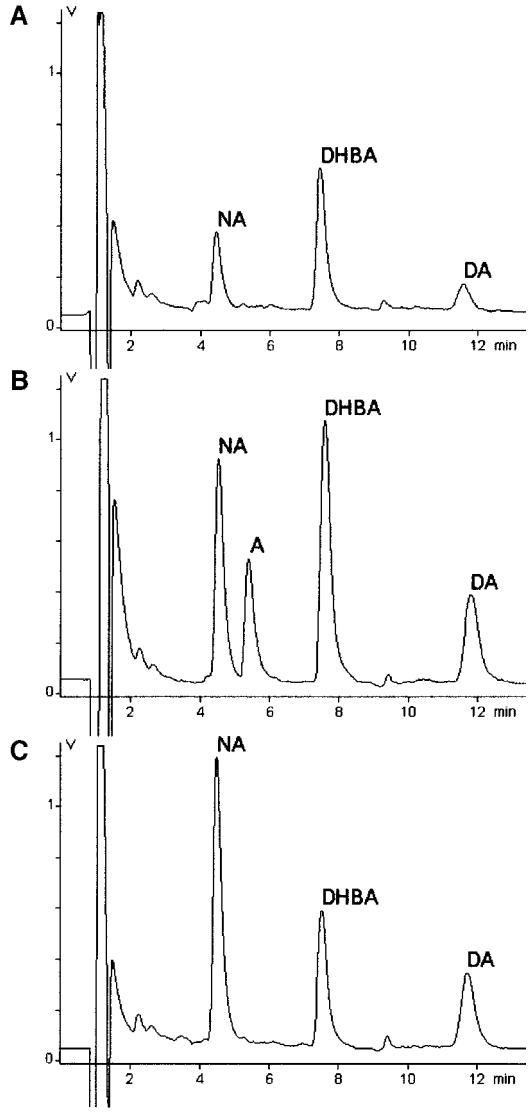

FIG. 1. HPLC–ED chromatograms of alumina extracts from oyster hemolymph samples and external standards. (A) Elution peaks for NA and DA extracted from the hemolymph of undisturbed oysters. (B) Elution peaks obtained from a mixture of an oyster hemolymph sample (identical to sample A) and an external standard solution containing NA, DHBA, A, and DA. (C) Elution peaks for NA and DA extracted from the hemolymph of oysters submitted to a 1-min shaking period. DHBA was used as an internal standard to determine recovery of catecholamines from hemolymph samples.

A temperature increase from 15° to 28°C also elicited significant ($P < 0.01$) CA responses (Fig. 5). Both NA and DA concentrations returned to basal levels 48 h after the beginning of the incubation at 28°C. In

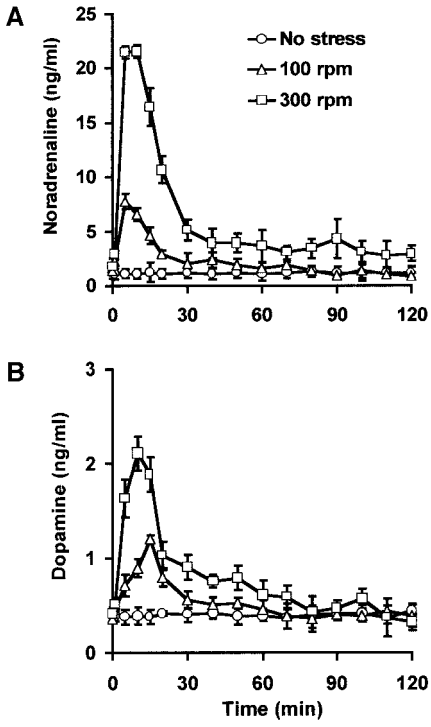

FIG. 2. The effect of acute stressors (consisting of shaking the animals on a laboratory agitator) of different intensities (100 or 300 rpm) on circulating noradrenaline (A) or dopamine (B) concentrations in oysters. Data are means and standard errors of three replicate experiments.

oysters subjected to a temperature decrease from 15° to 5°C, the CA response was smaller. Finally, a decrease in salinity (34 to 24 ppm) resulted in significant ($P < 0.01$) NA and DA responses (Fig. 6). Basal CA concentrations were reestablished 72 to 96 h after the beginning of the immersion in 24 ppm seawater. No oyster mortality was observed as a consequence of temperature or salinity changes during the experiments.

DISCUSSION

Changes in the physical environment (e.g., temperature, salinity, turbidity), organism interactions (competition for food, space, or sexual partners, predation, presence of parasites), and human interference, including aquaculture practices (handling, transport, sorting, grading) and pollution, constitute real stressors and cause stress hormone responses in vertebrates. Throughout their life cycle, invertebrates also face stressful situations that require physiological adaptations; however, little is known of the hormonal responses to stress in these animals. The present study has investigated the impact of stress on hemolymph CA concentrations in the oyster *C. gigas.*

Both NA and DA were detected in oysters' hemolymph, which is consistent with other studies showing that these CA and their biosynthetic enzymes are present in mollusc tissues (Sweeney, 1968; Hiripi *et al.*, 1977; Stefano and Catapane, 1980; Osborne, 1984; Rosza, 1984; Barraco and Stefano, 1990; Croll and Chiasson, 1990), including bivalves (Coon and Bonar, 1986; Osada and Nomura, 1989; Pani and Croll, 1995, 1998). This result is also consistent with previous work showing that NA and DA are present in mussel hemolymph (Dietz *et al.*, 1992) but it contradicts a recent study by Pani and Croll (2000), who reported an absence of NA in the hemolymph of the scallop *Placopecten magellanicus.* Considering that the extraction

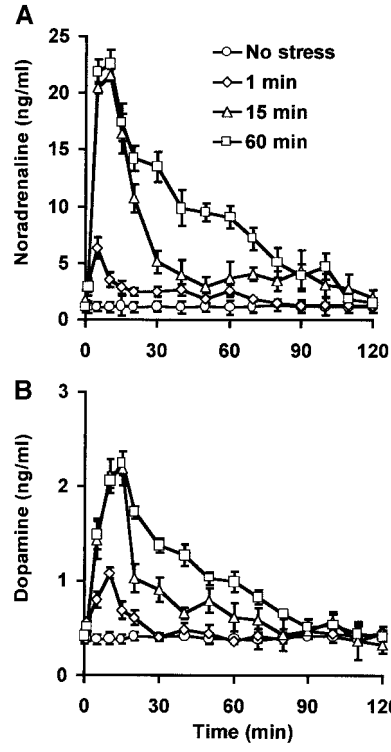

FIG. 3. The effect of acute stressors (consisting of shaking the animals at 300 rpm) of different durations (1, 15, or 60 min) on circulating noradrenaline (A) or dopamine (B) concentrations in oysters. Data are means and standard errors of three replicate experiments.

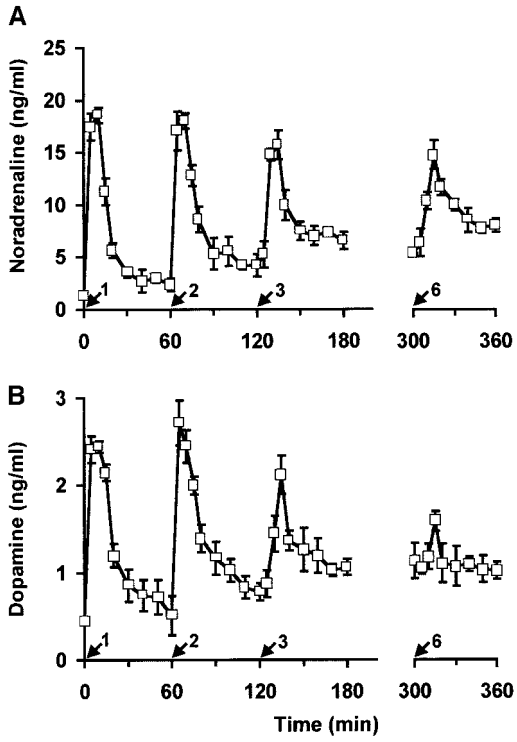

FIG. 4. The effect of repeated acute stress on circulating noradrenaline (A) or dopamine (B) in oysters. Oysters were submitted to six shaking periods (15 min each, 300 rpm) and left to rest for 45 min between each repetition. CA concentrations were measured during and after the first, second, third, and sixth repetitions. Data are means and standard errors of three replicate experiments. Arrows indicate starting times for the first, second, third, and sixth 15-min stress periods.

and detection methods used in our experiments are similar to those used by the above-cited authors, the discrepancy in the results remains unexplained. Measurable NA quantities (i.e., NA in a nonconjugated reduced form) decrease very rapidly in bivalve hemolymph. Indeed, in preliminary experiments where NA was added to oyster cell-free hemolymph at a final concentration of 18.0 ng/ml and kept at 15°C in a capped tube, measured NA quantities were 10.1 and $\leq$3.5 ng/ml, respectively, 20 and 50 min after the addition of NA and the estimated half-life time of detectable forms of NA in oyster cell-free hemolymph was estimated to be 25–30 min. NA has been reported to bind rapidly to vertebrate serum proteins (Powis, 1975a, b); thus, it is possible that this hormone also binds to oyster hemolymph proteins and becomes rapidly undetectable. Differences in hemolymph sample processing time may thus explain the discrepancy between the present results and those of Pani and Croll (2000).

Both NA and DA concentrations fell within the ranges reported for bivalve tissues (0.01–1.6 μg NA/g and 0.1–3.5 μg DA/g wet weight) and blood (1–20 ng NA/ml and 0.4–3 ng DA/ml) in previous studies (Osada and Nomura, 1989; Dietz *et al.*, 1992; Lacoste *et al.*, in press). Concentrations increased in response to acute and acute-repeated mechanical stress and fluctuated within ranges that induce physiological responses in bivalves (Muneoka and Kamura, 1982). These results and those of Werkman *et al.* (1990) suggesting that dopamine is involved in hormonal communication in molluscs indicate that the observed stress-induced CA changes could exert regulatory functions. Moreover, the CA response to stress in oysters reflected both the intensity and duration of the stressor. Indeed, shaking the animals for 1 min caused a relatively small rise in circulating CA, while 15 and 60 min of shaking elicited four- to fivefold higher CA levels that remained high until the end of the stimulus.

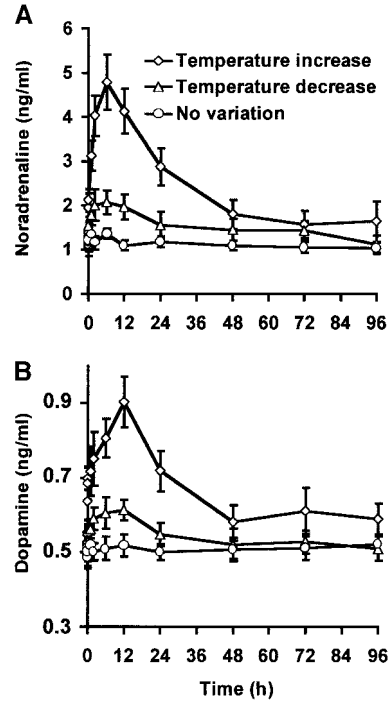

FIG. 5. Effect of temperature variations on circulating noradrenaline (A) or dopamine (B) concentrations in oysters. Temperature changes consisted of an increase from 15° to 28°C or a decrease from 15° to 5°C. Data are means and standard errors of three replicate experiments. Note time scale in hours.

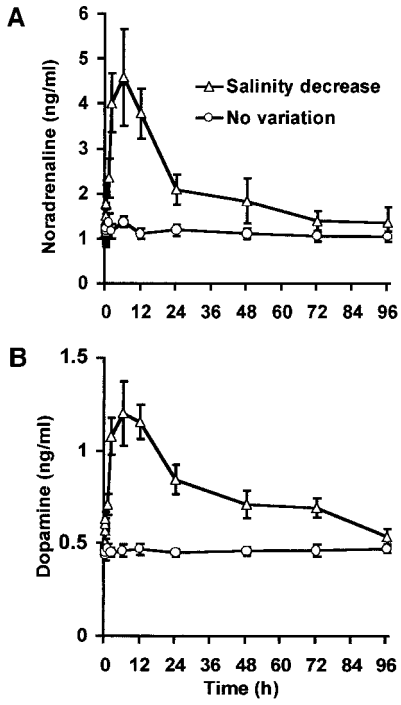

FIG. 6. Effect of a decrease in salinity from 34 to 24 ppm on circulating noradrenaline (A) or dopamine (B) concentrations in oysters. Data are means and standard errors of three replicate experiments. Note time scale in hours.

Following application of the stressor, CA were rapidly cleared from the hemolymph. The tissues and mechanisms that are responsible for the clearance of circulating CA in molluscs remain to be determined; however, previous studies have suggested the existence of CA degrading enzymes such as monoamine oxydase in bivalves (Pani and Croll, 1995, 1998, 2000). It is thus plausible that these enzymes are responsible for the return to basal CA concentrations in oysters.

When animals were subjected to repeated acute stress, the time necessary for CA concentrations to return to basal levels tended to increase, indicating lower CA clearance capacities in repeatedly stressed oysters. This phenomenon, which is also observed in mammals (McCarty *et al.*, 1997), was accompanied by a reduction in the amplitude of the hormonal response to stress, suggesting that sensitivity to CA increased or CA secretion capacities diminished in oysters submitted to repeated acute stress. Alternatively, habituation to stress, which has been observed in several vertebrates including fish (Schreck *et al.*, 1995), may occur in oysters and result in lessened CA responses.

Temperature or salinity changes also elicited long-lasting CA increases in oysters. These results are consistent with previous studies showing that temperature variations induce changes in monoamine concentration and metabolism in mollusc tissues (Hiripi *et al.*, 1977; Stefano *et al.*, 1978; Osada and Nomura, 1989). Moreover, Dietz *et al.* (1992) reported higher circulating DA levels in mussels subjected to salt-depleted water. Interestingly, CA are known to modulate the ciliary activity of gills (Aiello, 1990), the principal site of ion transport in bivalves (Dietz and Graves, 1981; Dietz *et al.*, 1982). Thus, it is possible that the CA changes induced by low salinity participate in the regulation of ion balance in oysters.

Previous studies have suggested that other biogenic amines such as octopamine probably play important roles in the stress response in insects and arthropods (Roeder, 1999; Hirashima *et al.*, 2000; Sneddon *et al.*, 2000; Stevenson *et al.*, 2000). Further work is thus needed to determine how stress-induced CA and other biogenic amine changes influence key metabolic mechanisms and physiological functions in molluscs. However, this study represents a further step in the characterization of the neuroendocrine stress–response system in these animals. The present results also offer the promise of useful tools for assessing the physiological status of bivalves in their natural habitat, under laboratory conditions, and in aquaculture settings.

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