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Role of biogenic amines and cHH in the crustacean hyperglycemic stress response

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Summary

In this study, we investigated (using bioassays and ELISA) the variation of cHH (crustacean hyperglycemic hormone) level in the eyestalks and hemolymph of *Palaemon elegans* (Rathke) (Decapoda, Caridea) following injection of serotonin (5-HT) and dopamine (DA) and correlated cHH profile with the variation in amount and time course of glycemia.

5-HT induced in *P. elegans* a rapid and massive release of cHH from the eyestalk into the hemolymph followed by hyperglycemia. On the contrary, DA did not significantly affect cHH release and hyperglycemia. In addition, we measured the level and variation of 5-HT in the eyestalk and hemolymph of *P. elegans* following copper contamination. The release of 5-HT from the eyestalk is very rapid and dose dependent. In the hemolymph, a peak of 5-HT occurs after 30 min, and again the circulating concentration of 5-HT is dose dependent on copper exposure. After 1 h, the level of 5-HT slowly decreases to basal level.

The release of 5-HT from the eyestalk into the hemolymph after copper exposure precedes the release of cHH, confirming its role as a neurotransmitter acting on cHH neuroendocrine cells. The fact that copper induced a rapid and massive release of 5-HT from the eyestalk can explain its demonstrated role in inducing the release of cHH and the consequent hyperglycemia in intact but not eyestalkless animals.

Key words: *Palaemon elegans*, copper, glucose, serotonin, dopamine, ELISA.

Introduction

Neuro-secretory structures in the eyestalk are the most important components of the neuroendocrine system of the stalk-eyed crustaceans. The hemolymph glucose concentration is controlled by the crustacean hyperglycemic hormone (cHH), a neuropeptide synthesized within the X-organ and released from the sinus gland (SG) complex, both of which are located in the eyestalk (Fingerman, 1992; Böcking et al., 2001). Hyperglycemia is a typical response of many aquatic animals to pollutants. In crustaceans, increased circulating cHH and hyperglycemia are reported to appear during exposure to several environmental stressors (Durand et al., 2000; Lorenzon et al., 1997, 2002, 2004b; Santos et al., 2001) in intact, but not eyestalkless animals, suggesting a cHH-mediated response (Fingerman et al., 1981; Reddy et al., 1994, 1996; Lorenzon et al., 2000). In the crab *Cancer pagurus*, emersion induced an increase in the hemolymph cHH after 4 h (Webster, 1996). Chang et al. (1998) monitored, by enzyme-linked immunosorbent assay (ELISA), the blood cHH variation in the lobster *Homarus americanus* following various environmental stresses. Emersion was a potent stimulator for elevation of cHH, while temperature and salinity variations were less effective. More recently, variation of cHH titer in the hemolymph was reported in the Norway lobster, *Nephrops norvegicus*, infected by the dinoflagellate parasitic *Hematodinium* sp. (Stentiford et al., 2001). Finally, increased water temperature induced an increment in blood cHH in *C. pagurus* and the crayfish *Procambarus clarkii* (Wilcockson et al., 2002; Zou et al., 2003).

Our recent study (Lorenzon et al., 2004b) demonstrated that, in the shrimp *P. elegans*, exposure to copper induced a dose-related rapid and massive release of cHH from the eyestalk into the hemolymph at the highest, lethal concentration, while a gradual and reduced discharge was revealed at the lowest concentration. The relationship between exposure to toxicant and release of cHH was confirmed by variation of blood glucose with a dose-related hyperglycemia that peaked 2 h after exposure to copper.

In order to understand the effect of stressors on hemolymph glucose, it is important to study the underlying hormonal mechanism. Biogenic amines and enkephalin have been found to mediate the release of several neurohormones from crustacean neuroendocrine tissues. Serotonin (5-HT; 5-hydroxytryptamine) is well known as a neurotransmitter in crustaceans on several grounds, and its levels have been measured in the nervous system and hemolymph of various crustacean species (Elofsson et al., 1982; Laxmyr, 1984;

Kulkarni and Fingerman, 1992), thus suggesting a possible role as a neurohormone (Rodrigues-Soza et al., 1997). Serotonin has long been known to have a potent hyperglycemic effect in several crustacean species (Bauchau and Mengeot, 1966; Keller and Beyer, 1968; Lee et al., 2000; Lorenzon et al., 1999, 2004a; Santos et al., 2001). Lee et al. (2001) confirmed the role of 5-HT in mediating the release of cHH. In the crayfish *Orconectes limosus*, injection of 5-HT caused a significant increase in the circulating level of cHH (Santos et al., 2001). In *P. clarkii*, cHH release increased as a dose-related function of 5-HT concentration (Escamilla-Chimal et al., 2002).

For dopamine (DA), studies on different species have reported contrasting results. In *P. clarkii* (Sarojini et al., 1995), *P. elegans*, the crayfish *Astacus leptodactylus* and the mantis shrimp *Squilla mantis* (Lorenzon et al., 1999, 2004a), DA inhibits the release of cHH from the SG, causing a decrease in hemolymph glucose level. Injection into eyestalkless animals is ineffective. By contrast, in the crab *Cancer maenas* (Lüschen et al., 1993) and in the tiger shrimp, *Penaeus monodon* (Kuo et al., 1995), DA was shown to elevate hemolymph glucose. More recently, Zou et al. (2003) demonstrated a dose-dependent effect of DA on the increase of cHH and glucose in the hemolymph of *P. clarkii*.

5-HT apparently plays a regulatory role in eyestalk hormone release, as has been previously demonstrated for MIH (molt inhibiting hormone), a neuropeptide belonging to the same family as cHH (Mattson and Spaziani, 1986), in the crab *Cancer antennarius* and by the recently described widespread occurrence of 5-HT_{1r} (type 1 serotonin receptor) in eyestalk ganglia (Spitzer et al., 2005).

In our previous work, we demonstrated in *P. elegans* that 5-HT has a marked dose-related effect in elevating glucose level but that it is ineffective in eyestalkless animals; DA injection in intact and eyestalkless animals produced a reduction below initial levels of hemolymph glucose (Lorenzon et al., 1999). The release of cHH and the consequent hyperglycemia is stress related, in particular after copper exposure (Lorenzon et al., 2004b). However, to our knowledge, there is a lack of information on the variation of 5-HT following stress and little connecting it with the hyperglycemic response.

As a consequence, the aim of this paper was to monitor the variation of cHH in the eyestalks and hemolymph of *Palaemon elegans* (Decapoda, Caridea) after injection of 5-HT and DA and relate it to the variation in amount and time course of blood glucose. Moreover, we intended to assess the level and variation of 5-HT in the eyestalk and hemolymph of *P. elegans* following copper contamination.

Materials and methods

Animal supply and maintenance

Specimens of *Palaemon elegans* (Rathke) (Decapoda, Caridea; 4–6 cm in length), a eurythermal and euryhaline species distributed widely along the coastal areas of Europe, were caught by cages in the Gulf of Trieste (Upper Adriatic Sea) and supplied by commercial fishermen.

They were stocked in 120 liter glass tanks with closed-circuit-filtered and thoroughly aerated 36‰ salinity artificial sea water (Prodac®, Padova, Italy) at 16–18°C and a natural L:D photoperiod at 300 lux intensity (type 49 fluorescent tube by Philips, Monza, Italy) during the light phase. They were fed *ad libitum* with bits of shrimp, cuttlefish or fish every second day; dead animals were removed daily.

Apparently healthy animals of both sexes and intermolt, having a body mass of 1–1.5 g, were used. Forty-eight hours before use, animals were housed individually in 500 ml plastic net cages immersed in larger tanks, for individual recognition. Animals were not fed during the experiment.

Hemolymph sampling and determination of glycemia

The animals were blotted dry and hemolymph (50 µl) was withdrawn, from the pericardial sinus, into sterile 1 ml syringes fitted with 25 g needles. Animals (N=10 for each treatment) were bled at 0 h, usually between 09.00 and 10.00 h to reduce possible interference due to circadian changes in blood glucose level (Kallen et al., 1990).

Hemolymph glucose content was quantified using a One Touch® II Meter (Lifescan, Miltipas, CA, USA) and commercial kit test strips (precision of strips ±3% coefficient of variation in the tested range). Due to the short time of processing, no anticoagulant was needed. In the results, variations of glycemia defined as increments are given as the mean of: {[(experimental value)/(value displayed by the same animal at 0 h)]–1}×100.

Effect of 5-HT and DA on blood glucose level

Variation of glycemia following injection with 5-HT and DA at the concentration of 10^{-8} mol g^{-1} live mass (concentration in the range previously tested by Lorenzon et al., 1999) was tested on groups of intact *P. elegans* (N=10 for each treatment). At 0 h, 0.5 h, 1 h, 2 h, 3 h and 24 h after injection, animals were bled as described above. A control group injected with saline was tested at the same time. Sterile saline for marine crustaceans was prepared with pyrogen-free distilled water and analytical-grade chemicals, according to Smith and Ratcliffe (1978), and autoclaved fro 25 min. All reagents were supplied by Sigma-Aldrich (St Louis, MO, USA).

Eyestalk homogenate and hemolymph treatment for ELISA of cHH and 5-HT variation

Groups of 10 *P. elegans* were injected with 5-HT or DA (10^{-8} mol g^{-1} live mass) or exposed to $CuCl_2$ (0.1 and 5 mg l^{-1} of Cu^{2+}); untreated and saline-injected animals were used as controls. Eyestalks were removed at time 0 h and then at 0.5 h, 1 h and 2 h (and 3 h only for Cu^{2+}) from the 10 animals of each different experimental group. Animals were anesthetized for 1 min on ice before ablation. The eyestalk was quickly frozen and the eyecup was cut off to remove visual and screening pigment. Eyestalk homogenate was prepared from 20 eyestalks homogenized in 2 ml of cold phosphate-buffered saline (PBS; Sigma), pH 8.0, and then centrifuged for 1 h at 930 ***g*** and 4°C.

The clear homogenate was quickly deep frozen at –20°C and stored until required for study.

Hemolymph was withdrawn from the different groups of 10 *P. elegans* for each treatment, as described above, at time 0 h and then at 0.5 h, 1 h and 2 h (and 3 h only for Cu^{2+}) and immediately centrifuged for 1 min at 10 300 ***g*** and 4°C to prevent coagulation. The supernatant plasma fraction was then stored at –20°C.

Direct ELISA of cHH

The ELISA was performed as described by Lorenzon et al. (2004b). 6×His-*Nen*cHHwt (M_r=11 kDa; Mettulio et al., 2004) recombinant protein was used as standard (y=1.49514–0.37828x; correlation coefficients of the fitted curves are >0.99 for all the data; r=0.9937, S.D.=0.44, P<0.001; Lorenzon et al., 2004b).

Briefly, 100 µl of the eyestalk homogenate (ES = eyestalk) or hemolymph from the different treatments, as well as standards, were loaded onto a 96-microwell plate (Costar, Bethesda, MD, USA) and incubated in duplicate overnight at 4°C. Then, 100 µl of the biotinylated anti-*Nen*cHH (anti *N. norvegicus* cHH; interspecific cross-reactivity tested by Giulianini et al., 2002) antibody (1 µg µl^{-1}), diluted 1:1000, was added to each well and the plate was incubated for 3 h at 36°C. After removal of the biotinylated antibody, plates were washed extensively, followed by the addition of 100 µl of streptavidin-peroxidase (Sigma) solution (diluted 1:5000), and incubated for 1 h at room temperature. The plates were once again washed and developed with 2,2′-azino-bis 3-ethylbenz-thiazoline-6-sulphonic acid solution (Sigma; liquid substrate ready for use) in darkness for 1 h at room temperature (100 µl per well).

The absorbance was measured in a multiwell plate reader (Anthos 2020, version 1.1; Anthos, Krefeld, Germany) at 405 nm.

Quantification of 5-HT in the hemolymph and eyestalk of P. elegans

A commercially available competitive serotonin ELISA kit (ICN Biochemicals, Costa Mesa, CA, USA) was used to determine the concentration of 5-HT in the hemolymph or eyestalk homogenate. The absolute value of 5-HT was obtained from the linear curve-fit of the standards. Sample values were then inserted into the equation and the amount of unknown 5-HT thereby determined (Fig. 1).

Statistical analysis

All statistics were performed using SPSS 9 (R) for Windows package (SPSS Inc., Chicago, IL, USA), and data are given as arithmetic means ± standard deviations. Effects of experimental treatments on blood glucose levels were analyzed. Analysis of variance (ANOVA) and Student's *t*-test were used to test the null hypotheses that all treatment means were equal and then all the data were tested by the LSD and Dunnett *post-hoc* test. The levels of significance were then calculated by Student's *t*-test for paired or independent data. A probability value of 0.05 or less of the statistical tests between the control and experimental values was considered significant. In order to test the statistical significance of responses to ELISA, experimental change values were compared using two-tailed Student's *t*-test, with the level of significance considered to be P<0.05. The time scale on graphs is not proportional, in order to achieve a better visual inspection of data and size of the illustration.

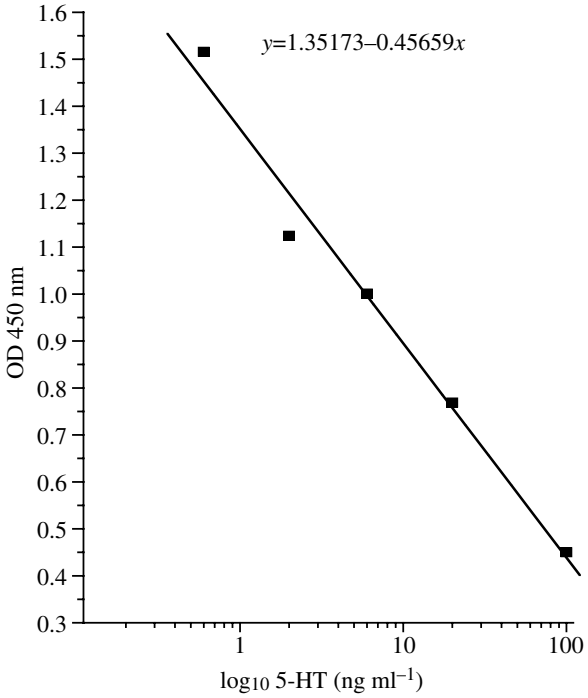

Fig. 1. A standard curve of five standards of 5-HT for ELISA. The x axis represents the $\log_{10}$ concentration value of the five standards. Absorbance was read in a multiwell reader at 450 nm. Correlation coefficient of the fitted line is r=0.9955 (S.D.=0.075, P<0.001).

Results

Effects of 5-HT and DA on the cHH content in the eyestalk and hemolymph and on the blood glucose level of P. elegans

Injection of 5-HT (10^{-8} mol g^{-1} live mass) induced a strong release of cHH from the eyestalk of *P. elegans* (Fig. 2). From 30 min after injection, the cHH content decreased drastically to 0.25±0.11 pmol ES^{-1} equiv. (ES equivalent), a value significantly different (P=0.001) from the initial value (5.60±2.6 pmol ES^{-1} equiv.) of untreated animals and from the saline-injected controls at the same time (5.41±1.86 ES^{-1} equiv.; P=0.001); values remained significantly (P<0.05) below the resting and control level throughout the experimental period. By contrast, DA (10^{-8} mol g^{-1} live mass; Fig. 2) produced no significant effect on cHH eyestalk content.

Fig. 3 shows hemolymph cHH variation after injection of the two bioamines. Following the massive release of cHH induced by 5-HT from the eyestalk, a rapid (less than 30 min) and marked increase of the circulating hormone was detected in the hemolymph, with a peak in cHH content of

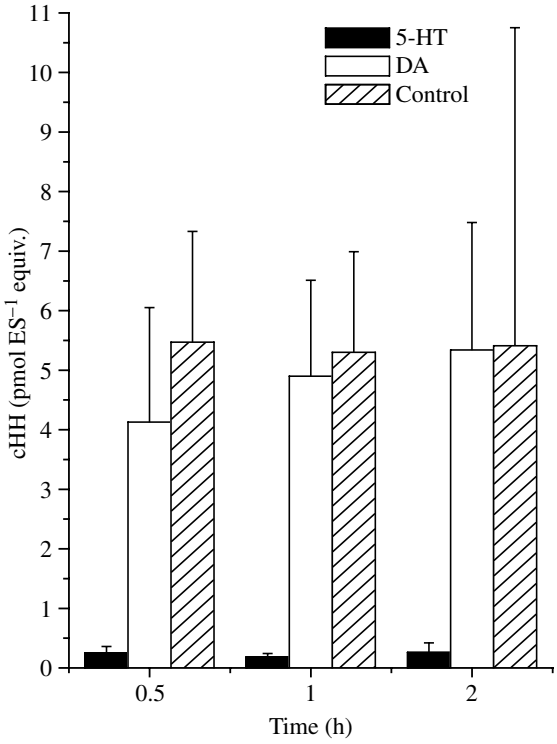

Fig. 2. Time course (0.5–2 h) of cHH in the eyestalk homogenates, each group pooled from 10 *P. elegans*, after injection of 5-HT or DA (10^{-8} mol g^{-1} live mass) and from the saline-injected control. Values are expressed as means ± S.D. (*N*=4).

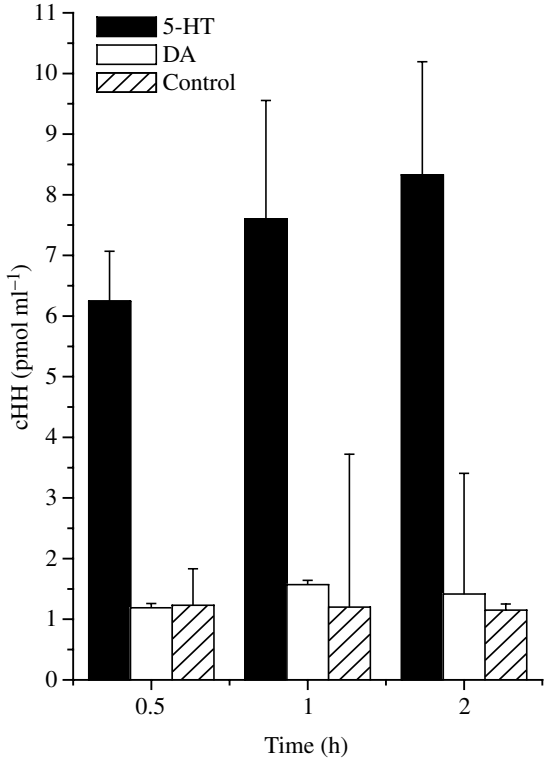

Fig. 3. Time course (0.5–2 h) of cHH in the hemolymph, each group pooled from 10 *P. elegans*, after injection of 5-HT or DA (10^{-8} mol g^{-1} live mass) and from the saline-injected control. Values are expressed as means ± S.D. (*N*=4).

8.33±1.87 pmol ml^{-1} (reached 2 h after injection), which was significantly different (*P*=0.001) from the initial untreated value (1.12±0.28 pmol ml^{-1}) and from the control value at the same time (1.13±0.05 pmol ml^{-1}; *P*=0.001). On the other hand, injection of DA (Fig. 3) did not significantly change hemolymph cHH throughout the time course.

Injection of 10^{-8} mol g^{-1} live mass of 5-HT (Fig. 4) induced a marked hyperglycemia, and blood glucose gradually rose from the first 30 min after injection to a peak at 2 h of 628±191% (63.90±1.91 mg dl^{-1}), which was significantly different from the initial value (9.20±1.93 mg dl^{-1}; *P*=0.001) and from the control at the same time, which showed an increase of 37±22% (12.90±2.56 mg dl^{-1}; *P*=0.001). Afterwards, the blood glucose value started to decrease and returned to the initial value after 24 h (data not shown). No significant (*P*>0.05 compared with the saline control) increase in blood glucose level (Fig. 4) was recorded in *P. elegans* after injection of DA (10^{-8} mol g^{-1} live weight).

5-HT but not DA is a strong stimulator of cHH release from the eyestalk into the hemolymph, and blood glucose variation after injection of the two bioamines follows the time course of hemolymph cHH.

Variation of 5-HT level in the hemolymph and eyestalk of P. elegans *following copper exposure*

Fig. 5 shows the time course of 5-HT in the hemolymph of *P. elegans* after exposure to Cu^{2+} (5 and 0.1 mg l^{-1}). In control

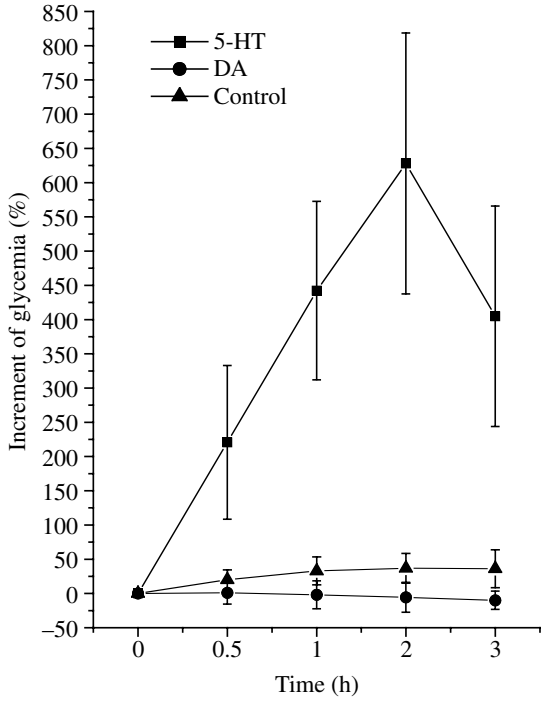

Fig. 4. Time course (0.5–3 h) of glycemia in the hemolymph of *P. elegans* after injection of 5-HT or DA (10^{-8} mol g^{-1} live mass) and in the saline-injected control group. Values of increment % are expressed as means ± S.D. (*N*=10).

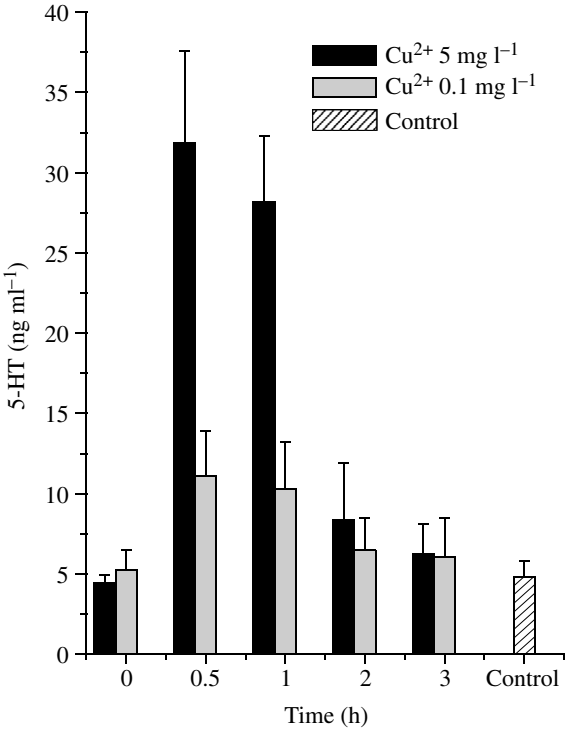

Fig. 5. Time course (0.5–3 h) of 5-HT in the hemolymph, each group pooled from 10 *P. elegans*, after exposure to different concentrations of Cu^{2+} and from the untreated control. Values are expressed as means ± S.D. (*N*=4).

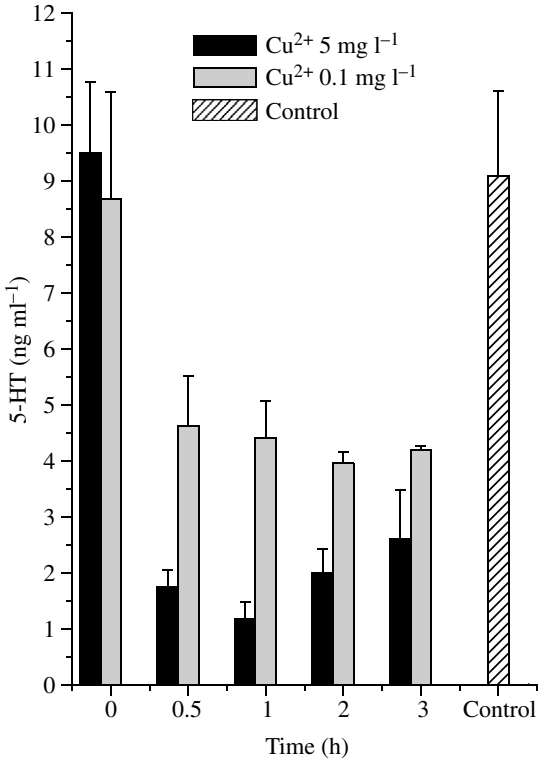

Fig. 6. Time course (0.5–3 h) of 5-HT in the eyestalk homogenates, each group pooled from 10 *P. elegans*, after exposure to different concentrations of Cu^{2+} and from the untreated control. Values are expressed as means ± S.D. (*N*=4).

animals, maintained in uncontaminated water, the level of circulating 5-HT was 4.85±0.98 ng ml^{-1}. The highest, lethal concentration (LC_{50}=3.27 mg l^{-1} at 96 h; Lorenzon et al., 2000) of 5 mg l^{-1} induced a marked and significant (*P*=0.001 *vs* initial value and control) increase of circulating 5-HT from the initial value of 4.41±0.51 ng ml^{-1} to 31.85±5.71 ng ml^{-1} in the first 30 min after exposure.

The value remained significantly elevated at 1 h (28.15 ng ml^{-1}), compared with the initial value and control (*P*=0.001), and decreased thereafter. At 2 h, the level of circulating 5-HT was 8.34 ng ml^{-1}, which was not significantly different from the initial value of 4.41 ng ml^{-1} and from the control (*P*>0.05). All further values were also not significantly different from the initial value and control (Fig. 5).

At the lower concentration of copper (0.1 mg l^{-1}; Fig. 5), the maximum increment in circulating 5-HT of 11.08±2.83 ng ml^{-1} was detected after 30 min of exposure and was significantly different from the initial value of 5.22±1.24 ng ml^{-1} (*P*=0.009) and from the control (*P*=0.019). The value obtained after 1 h (10.31±2.94 ng ml^{-1}) was still significantly different from the initial value (*P*=0.016) and from the control (*P*=0.012). Thereafter, the level of circulating 5-HT started to decrease and was not significantly different (*P*>0.05) from the initial value and from the control for the rest of the experiment.

Fig. 6 shows the time course of 5-HT level in the eyestalk of *P. elegans* after exposure to copper at a concentration of 5 mg l^{-1} and 0.1 mg l^{-1} and of untreated animals maintained in uncontaminated water and used as a control (resting level of 5-HT was 9.10±1.52 ng ml^{-1}). After exposure of *P. elegans* to 5 mg ml^{-1} of copper, a massive decrease of 5-HT was recorded; the level of 5-HT in fact fell from 9.51±1.26 ng ml^{-1} at time zero down to 1.17±0.31 ng ml^{-1} after 1 h, a value significantly different (*P*<0.05) from the initial control value. The level of 5-HT remained significantly (*P*<0.05) below the initial control value throughout the rest of the experiment.

At the lowest concentration of copper (Fig. 6), the level of 5-HT in the eyestalk decreased from 8.69±1.91 ng ml^{-1} to 4.63±0.89 ng ml^{-1} (*P*=0.02 *vs* initial value) in the first 30 min of exposure, which was also significantly different from the control (*P*=0.001). This value remained at the same level throughout the experimental period.

Discussion

The results presented in this study show that, in *P. elegans*, 5-HT induced a rapid and massive release of cHH from the eyestalk into the hemolymph, which was followed by an increase of blood glucose level. By contrast, DA did not exert any significant effect in releasing cHH, and no hyperglycemia was observed. This confirms our previous data on the same species (Lorenzon et al., 1999) on the effects of injection of 5-

HT, DA, L-enkephalin and their inhibitors on blood glucose levels. Moreover, these are the first measurements, to our knowledge, quantifying the variation of cHH in the eyestalk after a challenge with 5-HT and DA and following it throughout its effects in the blood compartment.

Our data accord with previous studies by Lee et al. (2001) that confirm the role of 5-HT in enhancing the release of cHH, which in turn elicits a hyperglycemic response in *P. clarkii*. Moreover, cHH release increases as a function of 5-HT concentration *in vivo*, and a similar trend was reported in an *in vitro* system by Escamilla-Chimal et al. (2002). In *O. limosus*, injection of 5-HT caused a significant increase of the circulating level of cHH (Santos et al., 2001).

Our present and previous data on the effects of DA demonstrate that, in *P. elegans*, *A. leptodactylus* and *S. mantis* (Lorenzon et al., 1999, 2004a), DA has no significant effect on the release of cHH and on the increase of hemolymph glucose level. Likewise, 5-HT and its receptor inhibitors, but not DA, affect serum ecdysteroid titer in intact *C. antennarius.* The lack of effect in eyestalk-ablated animals suggests a control on MIH release (Mattson and Spaziani, 1986).

Our findings confirm those by Sarojini et al. (1995) in *P. clarkii* but are in contrast with those by Lüschen et al. (1993) for *C. maenas*, Kuo et al. (1995) for *P. monodon* and Komali et al. (2005) in the freshwater prawn *Macrobrachium malcolmsonii*, where DA induced hyperglycemia. The present data on release of cHH are also at variance with those of Zou et al. (2003), who demonstrated in *P. clarkii* that DA induced the release of cHH and an increase in blood glucose level.

In the present paper, we also present the time course of 5-HT level in the eyestalk and hemolymph of *P. elegans* following exposure to different concentrations of copper. The circulating basal level of 5-HT in *P. elegans* was 4.81 ± 0.98 ng ml^{-1}, which is higher than that found by Sneddon et al. (2000) in *C. maenas*, where the basal level of 5-HT in the hemolymph was ~1 ng ml^{-1}, and different from those found in the crayfish *Pacifastacus leniusculus* (Elofsson et al., 1982).

The release of 5-HT from the eyestalk is very rapid (within 30 min) and dose dependent. In the hemolymph, a peak of increment is revealed after 30 min, and again the circulating concentration of 5-HT is dose dependent on copper exposure. After 1 h, the level of 5-HT slowly declines to the initial basal level.

In a previous work (Lorenzon et al., 2004b), we demonstrated the role of copper in inducing release of cHH from the eyestalk into the hemolymph, with a consequent increase in blood glucose level in a dose-related manner. The release of cHH from the eyestalk reached the maximum after 2 h of exposure, and in the hemolymph the maximum peak of circulating cHH was reached after 2 h of exposure. The release of 5-HT from the eyestalk (Elofsson et al., 1982; Kulkarni and Fingerman, 1992) into the hemolymph after stress precedes the release of cHH, confirming its role as a neurotransmitter acting on cHH neuroendocrine cells (Saenz et al., 1997; Garcia and Aréchiga, 1998; Escamilla-Chimal et al., 2002). The fact that copper induces the rapid and massive release of 5-HT from the eyestalk explains its demonstrated role in inducing the release of cHH (Lorenzon et al., 2004b) and the consequent hyperglycemia in intact but not in eyestalkless animals (Lorenzon et al., 2000). The basal level of cHH in the hemolymph of *P. elegans* is higher than in other species (Webster, 1996; Chang et al., 1998; Stentiford et al., 2001; Zou et al., 2003) and that could be related to the species or, in the presence of similar eyestalk cHH content (Lorenzon et al., 2004b) and rate of synthesis and release, to a smaller relative volume of the body fluid compartment and/or possibly to a slower turnover of the circulating hormone.

Therefore, further information is needed about the time course and detailed mechanisms that regulate the release of 5-HT and cHH and their binding to receptors, as well as about their half-life and catabolism in the hemolymph.

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