Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 446-452

CRF PROVOKES THE RELEASE OF NOREPINEPHRINE BY HEMOCYTES OF *Viviparus ater* (GASTROPODA, PROSOBRANCHIA): FURTHER EVIDENCE IN FAVOUR OF THE EVOLUTIONARY HYPOTHESIS OF THE "MOBILE IMMUNE-BRAIN"

E. Ottaviani, E. Caselgrandi*, A. Franchini, and C. Franceschi°

Department of Animal Biology, via Berengario, 14, *Institute of Hygiene, °Institute of General Pathology, University of Modena, 41100 Modena, Italy

Received April 23, 1993

Summary. The concentration of biogenic amines (norepinephrine, epinephrine and dopamine) was determined by HPLC in serum and hemocytes of the mollusc *Viviparus ater* following *in vitro* incubation of the hemolymph with corticotropin-releasing factor (CRF) for different periods of time. CRF provoked the release of norepinephrine from hemocytes into the serum, the maximum level being observed after 15 min. incubation. Moreover, immunoreactive tyrosine hydroxylase and dopamine ß-hydroxylase, i. e., the enzymes involved in biogenic amine biosynthesis, have been demonstrated in hemocytes. These findings show that in invertebrates an ancestral type of stress response, similar to that performed by mammalian hypothalamic-pituitary-adrenal axis, occurs in phagocytic hemocytes, a cell type capable of both fundamental immune and neuroendocrine responses.

In vertebrates the activation of the hypothalamic-pituitary-adrenal axis - the neuroendocrine marker of stress response - is dependent on the release of corticotropin-releasing factor (CRF). This phenomenon is crucial for the starting of a complex series of responses where the release of biogenic amines (norepinephrine, epinephrine and dopamine) play a fundamental role in maintaining body homeostasis (1).

Using different techniques, such as RIA tests, cytofluorimetric analysis and immunocytochemical procedures, we showed that CRF and pro-opiomelanocortin (POMC)-derived immunoreactive molecules, such as ACTH and ß-endorphin, are present in phagocytic hemocytes and serum of the mollusc *Planorbarius corneus* (2). Moreover, stress phenomena have been demonstrated in invertebrates (3-6). In particular, functional studies in *P. corneus* showed that the addition of CRF or ACTH to hemolymph was followed within 15 min. by an increased serum level of biogenic amines and a concomitant decrease of the same compounds in hemocytes (3). The hypothesis was put forward that this phenomenon may be an ancestral type of stress response occurring in hemocytes. We suggested that these molluscan cells, taken together,

0006-291X/93 $4.00

constitute an "mobile immune-brain" being capable of immune (chemotaxis, phagocytosis) (7, 8) and neuroendocrine responses (release of biogenic amines) (3), and containing a variety of neuropeptide-like molecules (9, 10).

In order to collect further data in favour of this hypothesis, experiments were performed in another mollusc, i.e. *V. ater*, whose hemocytes also contain POMC-derived molecules (11). We show here that the CRF-induced release of biogenic amines from hemocytes is a general phenomenon, and that hemocytes of *V. ater* contain the two key enzymes of the biogenic amines biosynthetic pathway.

Methods

Snails

Adult specimens of *Viviparus ater* (De Cristoferi & Jan, 1832) maintained in dechlorinated freshwater at room temperature were used.

Biogenic amine releasing procedures

The *in vitro* experiments were carried out on hemolymph samples collected as previously described (12) from various snails for each experiment. Each experiment was performed at least three times.

Sample preparation

Six ml of hemolymph were mixed with 600 µl of corticotropin-releasing factor (CRF) 10^{-7} M (Sigma, Chem. Co., St. Louis, USA) or snail saline solution (SSS) (13) in a plastic tube. The final concentration of CRF was 10^{-8} M. Samples were placed in the dark on a revolving mixer and incubated for a total of 45 min. at room temperature. At time 0, 15, 30 and 45 min., equal quantities (1.5 ml) were removed and centrifuged at 600 g. for 15 min. The supernatant (serum) and the pellet (hemocytes) were separated and the biogenic amine levels analyzed immediately.

Biogenic amine determination

Biogenic amine determination was performed as previously described (3). Briefly, 100 µl of SSS were added to the hemocyte pellet, and after shaking and centrifuging at 600 g. for 10 min. the supernatant was removed. This procedure was repeated twice in order to remove all traces of serum. The hemocytes were then homogenized with a Fisher Sonic dismembrator mod. 300 in 50 µl of 0.1 M $HClO_4$ containing 4 mM $NaHSO_3$. Samples were then centrifuged at 3000 g for 15 min and filtered with Millipore filters (type HV-0.45 µm). Finally, 40 µl of limpid acid supernatant were analyzed by direct injection into the chromatographic system. Components were identified and calculated by directly comparing areas of a standard calibration curve with those of the examined samples.

The catecholamines were extracted from the serum by absorption of activated alumina, followed by HPLC separation and assessment by electrochemical detection.

The Clin-Rep-Catecholamine kit allows the extraction of amines by the addition of 10 mg of activated aluminium oxide, 200 pg of DHBA (3, 4-dihydroxybenzylamine) (10 pg/µl) as an internal standard and 400 µl of 2 M tris buffer to 1.4 ml of serum. The serum was shaken for 15 min. and then centrifuged (1min). The supernatant was aspired and discharged. This procedure must be very accurate. The aluminium oxide was treated with 1 ml of a dilute buffer solution (0.2%) to wash away unwanted serum residue, centrifuged (1min) and again the supernatant was removed. The procedure was carried out three times. Finally selective desorption of the catecholamines was performed by acidic elution. An aliquot (100 µl) of the eluted acid comprising 100 µl of acetic acid, 50 µl of 10% sodium disulfite and 50 µl of 5% EDTA in 10 ml of H_2O was added to the sample, and the mixture was shaken for 15 min and centrifuged for 1 min. After removing the supernatant and filtering with a 0.45 µm Millipore filter, an aliquot (40 µl) of the eluted sample was directly injected into a Clin-Rep-HPLC column and the presence of an individual catecholamine tested by electrochemical detection.

The most reliable calculation of the quantitative analysis of catecholamines is obtained by the internal standard (DHBA) method with a correction factor.

Chemicals and reagents

The compounds investigated (norepinephrine, epinephrine, dopamine) and DHBA were obtained from Sigma, as various salts or free compounds. Stock solutions of 100 μg/ml of the individual compounds were prepared in 0.1 M $HClO_4$ containing 4 mM $NaHSO_3$. These were frozen and freshly prepared every 2 weeks. To obtain daily external standard mixture solutions and DHBA internal standard solutions, stock solutions were diluted with double distilled water to an appropriate concentration range (20 - 600 pg for epinephrine; 50 - 600 pg for norepinephrine and dopamine). The "Water Purification System Mill-Q (Millipore)" was used for the preparation of the solvents. The Clin-Rep-Catecholamine Kit was supplied by Pharma Vertriebs GmbH and Co KG - Munchen.

Liquid chromatography system

The HPLC Waters Catecholamines Analysis System, comprised of a Waters 590 Programmable Solvent Delivery Module and a Waters 460 Electrochemical Detector with a three-electrode amperometric cell, was used as previously described (14). The rate of flow was 1ml/min for 20 min. at room temperature.

Immunocytochemical procedures

The avidin-biotin peroxidase technique was performed on unfixed hemocytes as previously described (15). Anti-tyrosine hydroxylase (1: 1000) and anti-dopamine ß-hydroxylase (1:1000) polyclonal antibodies (Chemicon, Inter., Inc., USA) were used at 4°C for 24 h as the primary antibody. Controls treated with pre-immune sera were always negative.

Results

Morphological and cytofluorimetric studies have shown that the hemolymph of *V. ater* contains just one cell type: the spreading hemocyte. This cell belongs to the macrophage lineage, and is characterized by morphological and functional patterns typical of gastropod phagocytic cells (12).

The incubation of hemolymph with CRF 10^{-8} M for short periods of time provoked the release of norepinephrine into the serum, and its highest serum concentration was detected after 15 min. incubation (Fig. 1a). Concomitantly, a corresponding fall in hemocyte norepinephrine level was recorded (Fig. 1b). No significant release of epinephrine and dopamine was observed (Fig. 1a). Low concentration of epinephrine was detectable in hemocytes only at time 0 (3.5 ± 0.9 pg/10^6 cells). As expected, no significant changes in amine levels either in the serum or in the hemocytes were observed in hemolymph samples added with SSS (Figs. 2a, b). As far as dopamine is concerned, its level in CRF or SSS-treated hemocytes started decreasing with increasing time (Figs. 1b, 2b). The reason for this phenomenon is at present unclear. Spontaneous degradation of dopamine cannot be excluded.

Hemocytes treated with antisera to tyrosine hydroxylase (Fig. 3a) and dopamine ß-hydroxylase (Fig. 4a) were positive, whereas controls were always negative (Figs. 3b, 4b).

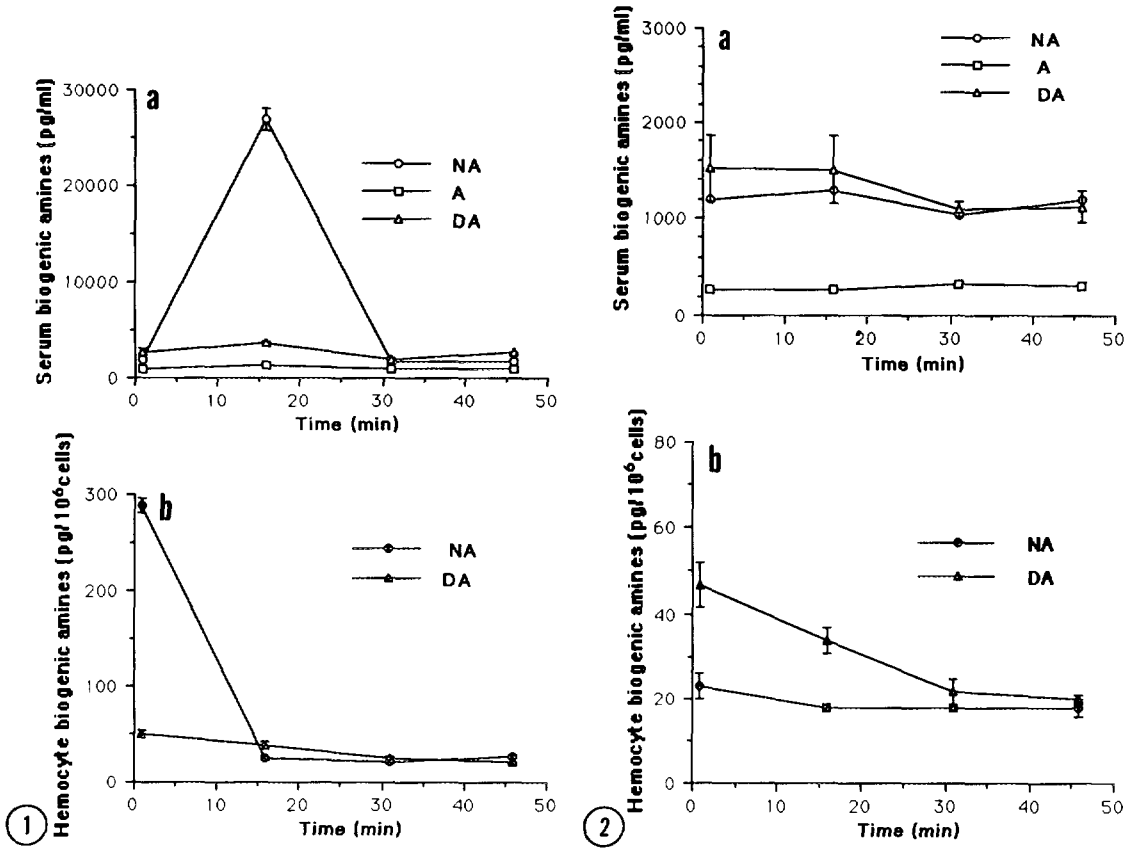

Fig. 1. Concentrations of biogenic amines in serum (**a**) and in hemocytes (**b**) of *V. ater* after incubation with CRF 10^{-8} M, determined by HPLC
NA = norepinephrine; A = epinephrine; DA = dopamine.
The determination of A was only possible at time 0: 3.5 ± 0.9 pg/10^6 cells.
The mean ± standard deviation of three experiments is shown.

Fig. 2. Concentrations of biogenic amines in serum (**a**) and in hemocytes (**b**) of *V. ater* after incubation with SSS, determined by HPLC
NA = norepinephrine; A = epinephrine; DA = dopamine.
The determination of A was only possible at time 0: 3.5 ± 0.9 pg/10^6 cells.
The mean ± standard deviation of three experiments is shown.

Discussion

In this paper we show that the addition of exogenous CRF to the hemolymph of *V. ater* induces the release of norepinephrine from cells into the serum. These results are similar to those previously described in *P. corneus* (3). In both molluscs, the maximum level of amine release was observed after 15 min. incubation, and values returned to their initial levels after 30 min. Moreover, in both mollusc species, norepinephrine was the most prominent amine released. These data further support the hypothesis that an ancestral type of stress response occurs in invertebrate hemocytes (3). We also demonstrated by immunocytochemical procedures that invertebrate hemocytes contain the two key enzymes of biogenic amine biosynthetic pathway, i.e. tyrosine hydroxylase

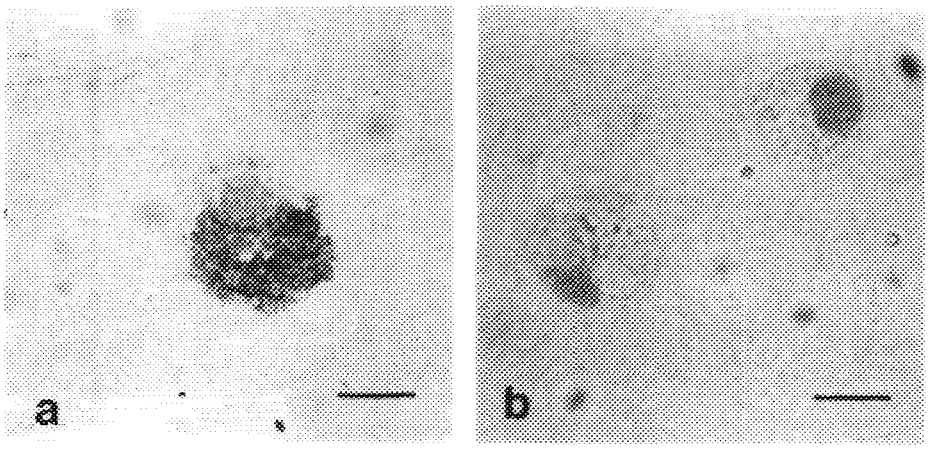

Fig. 3. Immunoperoxidase staining with tyrosine hydroxylase polyclonal antibody. Positive hemocyte (**a**) and negative control (**b**). The nuclei were counterstained with hematoxylin. Bar = 10 μm.

and dopamine ß-hydroxylase. The presence of these immunoreactive molecules suggests that the hydroxylation of tyrosine to DOPA, and of dopamine to norepinephrine probably occurs in *V. ater* hemocytes. Moreover, these results suggest that in molluscan hemocytes the synthesis of biogenic amines probably follows the same pathway observed in mammals (16).

As far as we know, this is the first demonstration of the presence of these enzymes in molluscan hemocytes.

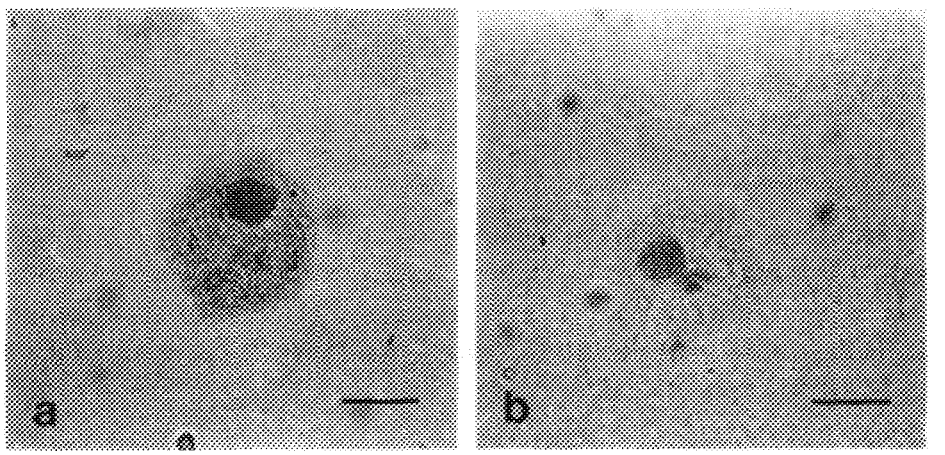

Fig. 4. Immunoperoxidase staining with dopamine ß-hydroxylase polyclonal antibody. Positive hemocyte (**a**) and negative control (**b**). The nuclei were counterstained with hematoxylin. Bar = 10 μm.

Taken together, these findings emphasize the great similarities of the events associated with CRF-induced release of biogenic amine in invertebrates and in vertebrates. However, in invertebrates all these phenomena occur in phagocytic hemocytes rather than in cells of the hypothalamic-pituitary-adrenal axis. These data confirm and extend the biological role of hemocytes, a cell type which in invertebrates substantially contribute to the maintenance of homeostasis. Throughout evolution, phagocytic hemocytes are fundamental in the most important defence mechanisms, such as phagocytosis and chemotaxis, besides containing ACTH and ß-endorphin (17). Moreover, hemocytes perform endocrine functions, such as those here reported, and contain a variety of molecules cross-reacting with antibodies to neurotransmitters and hormones (9, 10). Thus, from invertebrates to man, phagocytic cells are capable of both immune and neuroendocrine functions. These functions were divided later in evolution and were also performed by more specialized cells. This is most probably the evolutionary basis of the intense "cross-talk" between the immune and neuroendocrine systems, which is evident in more developed organisms including mammals (18, 19). The concept of the "mobile immune-brain" we have proposed in respect to the hemocyte of *P. corneus* (7), is confirmed and extended by the data presented in this paper. Indeed, these mobile cells are able to recognize a wide range of stimuli and to set up a complex response in which immune and neuroendocrine functions are interwoven.

Acknowledgment

This work was supported by a C.N.R. (Italy) grant to E.O. and C.F.

References

1. Axelrod, J., and Reisine, T.D. (1984) Science 224, 452-459.
2. Ottaviani, E., Petraglia, F., Montagnani, G., Cossarizza, A., Monti, D., and Franceschi, C. (1990) Regul. Pept. 27, 1-9.
3. Ottaviani, E., Caselgrandi, E., Petraglia, F., and Franceschi, C. (1992) Gen. Comp. Endocrinol. 87, 354-360.
4. Stefano, G.B., Cadet, P., Dokun, A., and Scharrer, B. (1990) Brain Behav. Immun. 4, 323-329.
5. Stefano, G.B., and Catapane, E.J. (1977) J. Pharmacol. Exp. Ther. 203, 449-546.
6. Stefano, G.B., Hiripi, L., and Catapane, E.J. (1978) J. Therm. Biol. 3, 79-83.
7. Ottaviani, E., Cossarizza, A., Ortolani, C., Monti, D., and Franceschi, C. (1991) Proc. R. Soc. Lond. [Biol.] 245, 215-218.
8. Ottaviani, E., Petraglia, F., Genedani, S., Bernardi, M., Bertolini, A., Cossarizza, A., Monti, D., and Franceschi, C. (1990) Ann. N.Y. Acad. Sci. 594, 454-457.
9. Ottaviani, E., and Cossarizza, A. (1990) FEBS Lett. 267, 250-252.
10. Ottaviani, E., Franchini, A., and Fontanili, P. (1992) Cell. Mol. Neurobiol. 12, 455-462.
11. Ottaviani, E., Caselgrandi, E., Franchini, A., and Franceschi, C. (1992) Abstract for the International Symposium on "Stress: The Neuropeptide Perspective", Rimini, Italy, September 6-9, 1992.

12. Ottaviani, E. (1989) J. Moll. Stud. 55, 379-382.
13. Ottaviani, E. (1983) Dev. Comp. Immunol. 7, 209-216.
14. Ottaviani, E., Caselgrandi, E., and Franchini, A. (1988) Comp. Biochem. Physiol. 89C, 267-269.
15. Ottaviani, E., Franchini, A., Cossarizza, A., and Franceschi, C. (1992) Neuropeptides 23, 215-219.
16. Stryer, L. (1988) Biochemistry. Freeman & Co., New York.
17. Ottaviani, E., Caselgrandi, E., Bondi, M., and Cossarizza, A., Monti, D., and Franceschi, C. (1991) Adv. Neuroimmunol. 1, 27-39.
18. Blalock, J.E. (1989) Physiol. Rev. 69, 1-32.
19. Khansari, D.N., Murgo, A.J., and Faith, R.E. (1990) Immunol. Today 11, 170-175.