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Effect of PDGF and TGF-β on the release of biogenic amines from invertebrate immunocytes and their possible role in the stress response

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Abstract PDGF-AB and TGF-β1 intervene in molluscan stress response, the former inhibiting and the latter inducing the release of norepinephrine and epinephrine from hemocytes. These amines are down-regulated even when TGF-β1 is added to hemolymph pre-incubated with PDGF-AB. The opposite behaviour is observed if the growth factors are reversed. The dopamine response is not affected in either case, even after the addition of CRH or ACTH. After pre-incubation with PDGF-AB or TGF-β1 in the presence of CRH or ACTH, norepinephrine and epinephrine release falls. These findings suggest that when the interaction is between growth factors, the order of combination is crucial, while in cases where the interaction is between growth factors and other peptides, such as CRH and ACTH, the order is of no importance.

Key words: Platelet-derived growth factor (PDGF)-AB; Transforming growth factor (TGF)-β1; Molluscan hemocyte; Biogenic amine; Stress response; HPLC

1. Introduction

The corticotropin-releasing hormone (CRH) – adrenocorticotropin hormone (ACTH) – biogenic amine axis is the main origin of stress response in vertebrates and in invertebrates (molluscs), even if there are differences in the manner in which the events associated to the release of biogenic amines takes place (see for review [1]). In invertebrates, the process is simplified, for rather than several organs, such as the hypothalamus, pituitary and adrenal glands, being involved, the response is concentrated in the phagocytic hemocytes, which harbour all the relevant molecules.

Furthermore, studies on the relationship between cytokines and biogenic amine release have shown that, as in vertebrates, cytokines are also involved in invertebrate stress response [2–4].

In this paper, we examine whether platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β1 are able to provoke the release of biogenic amines from molluscan hemocytes. It should be noted that the presence of PDGF- and TGFβ-like molecules in several invertebrate immunocytes, including molluscs, has been demonstrated [5]. Moreover, PDGF-AB and TGF-β1 are able to stimulate cell migration and enhance phagocytosis in molluscan hemocytes [6].

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2. Materials and methods

2.1. Animals

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

2.2. Sample preparation

About 35 ml of hemolymph were collected in a Pasteur pipette from 45 *V. ater*, by exerting pressure on the foot of each animal. The pooled hemolymph was divided into 11 portions (3 ml each one) and placed in plastic tubes. To 5 tubes the following substances were added: snail saline solution (SSS) (30 μl) (control sample) [7], platelet-derived growth factor (PDGF)-AB (Sigma Chem. Co., St. Louis, USA) (20 ng/ml final concentration), transforming growth factor (TGF)-β1 (Sigma) (5 pg/ml final concentration), human corticotropin-releasing hormone (CRH) (Sigma) (10^{-8} M final concentration) and human adrenocorticotropin hormone (ACTH) (Sigma) (10^{-8} M final concentration). The concentrations used of the above-mentioned peptides are active on molluscan hemocytes [1,6]. Of the 6 remaining tubes, 3 were pre-incubated with PDGF-AB for 2 h before addition of TGF-β1, CRH or ACTH, while the other 3 were pre-incubated with TGF-β1 for 2 h before adding PDGF-AB, CRH or ACTH. The tubes were then placed in the dark on a revolving mixer, incubated for 20 min at room temperature and immediately centrifuged ($600 \times g$ for 15 min). After centrifugation, the supernatant (serum) and the pellet (hemocytes) were collected, and the amount of biogenic amines [norepinephrine (NA), epinephrine (A), dopamine (DA)] in the serum was immediately determined by liquid chromatography.

2.3. Determination of biogenic amines

The serum was analyzed using a Clin-Rep-Catecholamine kit, which involves the extraction of biogenic amines by activated aluminium oxide and the de-absorption of the catecholamines with acid solution. 50 μl of a solution of DHBA (3,4-dihydroxybenzylamine) (10 pg/μl) as an internal standard, 10 mg of aluminium oxide, and 400 μl of 2 M Tris buffer, pH 8.7, were added to 1.4 ml of serum. The mixture was shaken for 15 min in the dark at room temperature and centrifuged (1 min), and the supernatant was aspirated and discarded. One ml of Tris buffer, pH 8.1 (0.2%), was added to the remaining sample, shaken for 1 min and then centrifuged (1 min). The supernatant was removed and discarded and the sample was washed 3 times. To elute catecholamines, the remaining sample was mixed with 100 μl of eluting acid solution (100 μl of 100% acetic acid, 50 μl of 10% sodium disulfite and 50 μl of 5% EDTA in 10 ml of ultrapure water), shaken for 15 min in the dark at room temperature and then centrifuged for 4 min. The supernatant was removed with care, filtered with a 0.45 μm Millipore filter and injected (50 μl) directly into the chromatographic system. This acid solution was stable for 2 days at 4°C, for 15 days at −20°C and for at least 2 months at −80°C. We investigated the stability of biogenic amines in serum and after extraction. Our results suggest that it is better to carry out extraction immediately and store the acid solution if is not possible to do the chromatographic analysis. The most reliable calculation of the quantitative analysis of catecholamines was obtained using the internal standard (DHBA) method with a correction factor.

2.4. Chemical reagents

The substances investigated (NA, A, DA) and DHBA were obtained from Sigma as salts or free compounds. Stock solutions containing 100 μg/ml of the individual compounds were prepared in 0.1 M

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Table 1
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Viviparus ater* after addition of 20 ng/ml PDGF-AB, 5 pg/ml TGF-β1, 10^{-8}M CRH or 10^{-8}M ACTH

	Saline	PDGF-AB	TGF-β1	CRH	ACTH
NA	436.00 ± 67.48[a]	130.00 ± 20.98[b]	634.00 ± 96.59[c]	795.00 ± 225.12[c]	679.00 ± 35.08[b]
A	705.00 ± 186.70	371.00 ± 100.35[c]	1673.00 ± 280.31[b]	712.00 ± 352.78	1071.00 ± 142.66[c]
DA	1337.00 ± 458.56	1210.00 ± 156.72	1195.00 ± 100.41	1448.00 ± 245.26	1368.00 ± 464.17

NA, norepinephrine; A, epinephrine; DA, dopamine.
[a]The mean ± standard deviation of three experiments is shown. Data are expressed as pg/ml.
Statistical analysis was performed by Student *t*-test. [b]$p < 0.01$ and [c]$p < 0.05$ vs. saline (control).

$HClO_4$ containing 4 mM $NaHSO_3$. These were kept frozen and freshly prepared every 2 weeks. Daily external standard mixture solutions and DHBA internal standard solution were obtained by diluting stock solution to the working concentration with bi-distilled water. The water used for preparing standards and solvents was obtained using a Milli-Q Water Purification System (Millipore). The Clin-Rep-Catecholamine kit was provided by RECIPE Pharma Vertriebs GmbH and Co. KG, München, Germany.

2.5. Liquid chromatography system

The HPLC apparatus consists of a Isocratic LC Pump (mod. 250 Perkin Elmer) equipped with a degasser (ERC-3312-Erma), an automatic injector Rheodyne 7125 (50 μl loop) and a Waters 460 electrochemical detector employing a three-electrode amperometric cell. The potential of the working electrode was maintained at +0.65 V vs. a silver/silver chloride reference electrode. The signal was recorded and elaborated with a Data System PE Nelson (mod. 1020 Perkin Elmer). Analysis was performed in isocratic mode at room temperature and at a flow rate of 1.2 ml/min, and lasted for 8 min. The mobile phase and the column employed were from the catecholamine kit purchased from RECIPE Pharma Vertriebs GmbH and Co. KG.

2.6. Statistical analysis

Student's two-tailed *t*-test was used to compare the biogenic amine release of control (presence of SSS) and treated samples (presence of PDGF-AB, TGF-β1, CRH, ACTH), as well as to compare controls and samples obtained after pre-incubation of the hemolymph with the two growth factors. The results with PDGF-AB, TGF-β1, CRH or ACTH alone were also compared with those obtained after pre-incubation with one of the growth factors followed by the addition of CRH, ACTH or the other growth factor, respectively.

3. Results

The results reported in Table 1 show a significant decrease with respect to controls in the release of NA and A from hemocytes in the presence of PDGF-AB, while TGF-β1 was able to provoke a significant increase in these amines in the serum. Moreover, TGF-β1 was able to provoke the maximum release of A into the serum. Neither growth factor significantly affects DA release.

As expected, both CRH and ACTH were able to induce the release of biogenic amines.

The situation changes when the hemolymph is pre-incubated with the two growth factors. As shown in Table 2, pre-incubation with PDGF-AB followed by the addition of TGF-β1, CRH or ACTH results in a general, significant decrease in the level of biogenic amines with respect to values following incubation of the hemolymph with the single molecules (TGF-β1, CRH, ACTH) (Table 1). NA concentrations following pre-incubation with PDGF-AB cannot be determined, because values were below the sensitivity threshold of the method. The concentration of A shows a significant decrease ($p < 0.01$) with respect to incubation with TGF-β1 alone. The levels of DA are not significantly different from those obtained in the presence of TGF-β1, CRH and ACTH used singularly (Table 1).

Pre-incubation with TGF-β1 (Table 3) followed by the addition of PDGF-AB shows a significant increase in NA ($p < 0.01$), but no significant modification of the A and DA concentration with respect to incubation with PDGF-AB alone (Table 1). The addition of CRH or ACTH provokes a significant decrease in NA ($p < 0.01$) and a lower release of A, but again DA values not significantly different from those found when the hemolymph is incubated only with either CRH or ACTH (Table 1).

4. Discussion

We have shown that PDGF-AB and TGF-β1 are involved in different ways in the release of biogenic amines (NA, A, DA): PDGF-AB shows an inhibitory effect, while TGF-β1 can induce amine release. Moreover, as expected, CRH and ACTH also intervene in the release of biogenic amines. These most recent results reinforce our previous observations on stress response in molluscs [1,8]. We have demonstrated both that the key mediator molecules in molluscs are similar to those present in vertebrates, and that the pathway follows the same order and pattern, i.e. CRH, ACTH, and biogenic amines. In this perspective, the growth factors also play an important role in the molluscan stress response. Either directly or indirectly, they are able to up- or down-regulate the release of biogenic amines, and this phenomenon is particularly evident for NA and A. Moreover, the relationship between the two growth factors should be underlined. Pre-

Table 2
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Viviparus ater* following addition of 5 pg/ml TGF-β1, 10^{-8}M CRH or 10^{-8}M ACTH after pre-incubation with 20 ng/ml PDGF-AB

	Saline	TGF-β1	CRH	ACTH
NA	436.00 ± 67.48[a]	b.s.	b.s.	b.s.
A	705.00 ± 186.70	502.00 ± 94.69	394.00 ± 116.99	530.00 ± 55.25
DA	1337.00 ± 458.56	1151.00 ± 270.49	1399.00 ± 347.36	1330.00 ± 99.43

NA, norepinephrine; A, epinephrine; DA, dopamine. b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. Statistical analysis was performed by Student *t*-test.
[a]The mean ± standard deviation of three experiments is shown. Data are expressed as pg/ml.

Table 3
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph (serum) of *Viviparus ater* following addition of 20 ng/ml PDGF-AB, 10^{-8}M CRH or 10^{-8}M ACTH after pre-incubation with 5 pg/ml TGF-β1

	Saline	PDGF-AB	CRH	ACTH
NA	436.00 ± 67.48[a]	481.00 ± 59.09	b.s.	351.00 ± 62.06
A	705.00 ± 186.70	383.00 ± 16.26[b]	415.00 ± 391.00	674.00 ± 330.00
DA	1337.00 ± 458.56	1085.00 ± 211.06	1437.00 ± 327.65	1463.00 ± 503.57

NA, norepinephrine; A, epinephrine; DA, dopamine. b.s., below the sensitivity of the HPLC assay. The detection limit is 20 pg. Statistical analysis was performed by Student *t*-test.
[a]The mean ± standard deviation of three experiments is shown. Data are expressed as pg/ml. [b]$p < 0.05$ vs. Saline (control).

incubation with the inhibitor PDGF-AB annuls the inducing power of TGF-β1. While following pre-incubation with TGF-β1, PDGF-AB partially loses its inhibitory effect on the release of biogenic amines, i.e. NA. DA, however, is not affected, and, we obtained the same DA values either with PDGF-AB and TGF-β1 used singularly, or when the hemolymph was pre-incubated with one growth factor before the addition of the other. Similar behaviour is observed after addition of CRH or ACTH.

Therefore, the behaviour of PDGF-AB and TGF-β1 with regard to CRH is similar to that observed with cytokines [3,4]. Indeed, using the same experimental approach, IL-2 was able to elicit the release of biogenic amines from hemocytes, but this response was significantly reduced when the hemolymph was incubated with CRH after pre-incubation with IL-2. This inhibitory effect was also observed with IL-1α, IL-1β, TNF-α and TNF-β.

This study demonstrates that the two growth factors act differently when variously combinated with other substances. If the interaction is between growth factors, the order of combination is crucial, while this order is not important when interaction is between growth factors and other peptides. It should be emphasized that previous studies have shown that the action of a growth factor may depend on several parameters, such as the presence of other substances and the differentiation of target cells, etc. [9]. Indeed, TGF-β stimulates the growth of some fibroblasts in the presence of PDGF, while the action is reversed in the presence of epidermal growth factor [10]. Moreover, PDGF acts in synergy with TGF-β in stimulating DNA synthesis in sparse cultures of human embryonic fibroblasts [11].

On the whole, our data confirm from a phylogenetic point of view reports in the literature on mouse and man. Cytokines and growth factors are part of a common pool of polypeptides with regulatory function [12]. This and previous studies suggest that these substances also have overlapping and distinct biological properties in invertebrates. They are present in the same immunocytes, i.e. cells with phagocytic activity [5], are able to influence hemocyte migration and increase phagocytic capacity [6] and can intervene in the stress response with either an inhibiting or inducing effect.

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