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The effect of corticotropin-releasing factor and pro-opiomelanocortin-derived peptides on the phagocytosis of molluscan hemocytes

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Abstract. The effect of corticotropin-releasing factor (CRF) and pro-opiomelanocortin (POMC)-derived peptides on hemocyte phagocytosis in two molluscs, *Planorbarius corneus* and *Viviparus ater* was studied. The peptides and related fragments examined are those which have been shown to influence hemocyte motility in the two species. The results obtained revealed that the effects on phagocytosis are not directly correlated with previous findings on cell motility. Furthermore, the mode of action of an individual peptide could be species-specific and dose-dependent. The relationships between peptides, locomotion and phagocytosis in these molluscs are discussed.
Key words. Phagocytosis; corticotropin-releasing factor (CRF); pro-opiomelanocortin (POMC)-derived peptides; hemocytes; molluscs.

Using immunocytochemical, cytofluorimetric and RIA procedures, we and others have demonstrated the presence of corticotropin-releasing factor (CRF) and pro-opiomelanocortin (POMC)-derived peptides in the blood cells of invertebrates and vertebrates[1–4]. These results have also been confirmed by in situ hybridization using a human POMC probe. Indeed, both invertebrate and vertebrate blood cells express POMC-mRNA, proving that the cells contain the gene which codes the POMC-derived peptides, and that this gene is highly conserved[5]. These peptides, in particular those that are involved in cell migration, phagocytosis and stress response, are known to play a role in the modulation of the immune system[6–11]. As far as cell migration is concerned, molluscan hemocyte locomotion is stimulated by ACTH-(1-24), by shorter ACTH fragments, by the whole β-endorphin sequence, by its N- and C-terminal fragments or the (2–17) sequence (which lacks both N- and C-terminals), and by N-acetylated β-endorphin. Naloxone can partially reverse the effect of endorphin[6,7]. Moreover, in vitro studies have demonstrated that the hemocytes of *Planorbarius corneus* are able to release ACTH-like molecules into the hemolymph, and that the release of these endogenous molecules provokes in turn the release of biogenic amines from the hemocytes themselves[9]. In vitro bacterial phagocytosis tests using ACTH-(1–24) at different concentrations (10^{-8}–10^{-17} M) reveal a dose-dependent increase in the phagocytic activity of the hemocytes, an increase which continues down to a concentration of 10^{-13} M[10]. Finally, β-endorphin-(1–31) does not influence biogenic amine release, nor is it able to enhance bacterial phagocytosis[12].

The aim of the present paper is to assess the possible influence on phagocytosis of CRF, ACTH, β-endorphin and related fragments which have been shown to affect hemocyte motility in *P. corneus* and *V. ater*.

Materials and methods

Hemolymph from two molluscs, *Planorbarius corneus* (L.) and *Viviparus ater* (De Cristofori and Jan, 1832), was collected as previously described[13,14]. In vitro bacterial phagocytosis was carried out by adding 100 µl of *Staphylococcus aureus* suspension (10^6–10^8 bacteria/ml) and 100 µl of different peptide solutions (10^{-7} M and 10^{-10} M) to 800 µl of hemolymph. Drops of the mixture (100 µl) were placed on a slide, to which an adhesive PVC strip, in which two 10 mm diameter holes had been made, was attached. The following peptides were used: Corticotropin-releasing factor (CRF); ACTH- (1–24), -(1–4), -(4–9), -(1–13), -(4–10), -(1–17), -(11–24); endorphin-(1–31), -(6–31), -(18–31), -(1–17) (Sigma Chemical Co., St. Louis, Missouri, USA) (final concentrations: 10^{-8} and 10^{-11} M). The CRF phagocytosis tests were also carried out with α-helical CRF (10^{-6} M) (Peninsula Lab. Inc., Belmont, California, USA). Incubation was performed in a humified chamber, and phagocytosis was estimated after 30 min. The specimens were rinsed in snail saline solution (SSS)[13] and stained with toluidine blue, and the number of phagocytized bacteria in 20 randomly selected hemocytes in each preparation was recorded under the microscope. Phagocytosis tests were repeated four times for each peptide. Student's two-tailed t-test was used to compare phagocytosis in treated and control (in the presence of SSS) samples.

Results

The findings of the in vitro phagocytosis tests reported in table 1 show that in *P. corneus* the peptides used did not influence phagocytosis. On the other hand, various effects were observed in *V. ater* (table 2). With

Table 1. Influence of CRF, ACTH and endorphin on in vitro phagocytosis of *P. corneus* hemocytes.

Peptide	Controls	Treated	
		10^{-8} M	10^{-11} M
CRF	5.11 ± 4.09	5.22 ± 2.25	5.13 ± 2.07
CRF + α-helica CRF	5.11 ± 4.09	5.59 ± 2.74	5.13 ± 1.56
ACTH-(1–24)	-	§	§
ACTH-(1–4)	4.97 ± 2.09	5.22 ± 1.89	5.11 ± 1.66
ACTH-(4–9)	4.97 ± 2.09	4.41 ± 2.34	5.04 ± 1.89
ACTH-(1–13)	4.97 ± 2.09	5.17 ± 2.74	4.81 ± 2.18
ACTH-(4–10)		n.d.	n.d.
ACTH-(1–17)		n.d.	n.d.
ACTH-(11–24)	4.97 ± 2.09	4.99 ± 2.25	4.65 ± 1.79
endorphin-(1–31)		§	§
endorphin-(6–31)		n.d.	n.d.
endorphin-(18–31)	4.62 ± 1.68	5.06 ± 2.46	4.89 ± 1.98
endorphin-(1–17)	4.62 ± 1.68	4.87 ± 1.56	4.14 ± 1.26

Data are means ± SD of bacteria/hemocyte.
§ = previously tested peptides: ACTH-(1–24) = positive; β-endorphin-1(1–31) = negative[9,11].
n.d. = not determined because had no effect on cell motility[6,7].

Table 2. Influence of CRF, ACTH and endorphin on in vitro phagocytosis of *V. ater* hemocytes.

Peptide	Controls	Treated	
		10^{-8} M	10^{-11} M
CRF	6.74 ± 3.22	9.36 ± 4.48*	9.66 ± 3.74*
CRF + α-helica CRF	6.74 ± 3.22	7.20 ± 3.06	7.13 ± 2.76
ACTH-(1–24)	4.60 ± 2.02	7.77 ± 3.36*	7.20 ± 2.99*
ACTH-(1–4)	4.60 ± 2.02	5.70 ± 2.31**	6.05 ± 2.97**
ACTH-(4–9)	4.60 ± 2.02	4.85 ± 1.98	4.99 ± 2.23
ACTH-(1–13)		n.d.	n.d.
ACTH-(4–10)	4.60 ± 2.02	4.53 ± 1.79	5.61 ± 2.62***
ACTH-(1–17)	6.83 ∓ 3.13	6.67 ± 2.71	6.21 ± 2.64
ACTH-(11–24)		n.d.	n.d.
endorphin-(1–31)	6.83 ± 3.13	7.47 ± 4.30	6.90 ± 4.00
endorphin-(6–31)	6.83 ± 3.13	6.90 ± 2.83	6.37 ± 2.81
endorphin-(18–31)	6.83 ± 3.13	7.13 ± 2.97	6.90 ± 2.97
endorphin-(1–17)	6.83 ± 3.13	7.03 ± 3.24	7.06 ± 2.83

Data are means ± SD of bacteria/hemocyte.
*$p < 0.001$, **$p < 0.005$, ***$p < 0.05$.
n.d. = not determined becaused had no effect on cell motility[6,7].

CRF, hemocyte phagocytosis increased significantly ($p < 0.001$) at both concentrations used; however, pre-incubation of CRF with a specific inhibitor, i.e. α-helical CRF, eliminated this stimulation. As far as ACTH fragments were concerned, ACTH-(1–24) and ACTH-(1–4) significantly increased phagocytic activity at both the concentrations used, while ACTH-(4–10) was effective only at the lower concentration (10^{-11} M). The whole β-endorphin molecule as well as its fragments had no effect on phagocytosis.

Discussion

All the peptides studied for their effect on phagocytosis have also been shown to influence hemocyte motility in one or both of the species (*P. corneus* and *V. ater*), and this influence was found at the same concentrations as used here (10^{-8} and 10^{-11} M)[6,7]. A comparison of the results of the phagocytosis and cell motility tests clearly shows that no direct correlation exists between the effect on cell motility and that on phagocytosis, as the peptides which influence cell motility do not always have an effect on phagocytosis. Furthermore, the data suggest that, as already found in the cell motility experiments[6,7], the effect on phagocytosis of an individual peptide could also be species-specific and dose-dependent. Indeed, a single peptide may be able to modulate the immune functions of one species not those of another. The same observations would be true with respect to the concentrations at which the peptides are used. At present, it is difficult to understand the way in which these peptides act on cell motility and phagocytosis. For example, in *V. ater*, the ACTH-(1–4) fragment influences both hemocyte motility and phagocytosis at both concentrations used, while in *P. corneus* it is effective only on cell motility at the higher concentration[7]. This behaviour could be attributed to the fact that fragments are less complex than the entire molecule. Indeed, the effects of ACTH-(1–24) and of the entire β-endorphin molecule would appear to be uniform, acting on both species: ACTH-(1–24) influences hemocyte motility and enhances phagocytosis[10], while β-endorphin stimulates only cell motility[6,7]. However, this behaviour is in complete contrast to that of CRF, which affects motility and phagocytosis in *V. ater*, but elicits no response in *P. corneus*. Moreover, with a further species, *Mytilus edulis*, CRF causes immunosuppression[15].

The means by which ACTH, β-endorphin and related fragments influence cell activity remains unclear, particularly in invertebrates. Initially, the peptides have to find an appropriate receptor on the cell. However, apart from the report of a specific receptor for opioids in *M. edulius* hemocytes[16], few studies have considered this issue in invertebrates. Even less data is available on the transduction of the signal after ACTH or opioid interaction with the cell membrane. Our previous results suggest that the cell response provoked by ACTH-(1–24) in the hemocytes of *P. corneus* and *V. ater* could be mediated by cAMP[17,18].

It is generally accepted that phagocytosis in macrophages is initiated by the stimulation of a chemotactic response to foreign particles, while the process is enhanced by opsonic factors which augment phagocytosis by coating foreign particles. This phenomenon has also been demonstrated in gastropods[19–24]. A vast range of foreign materials, such as bacteria, latex particles, dead hemocytes, etc. are phagocytized both in vivo and in vitro by hemocytes which present several typical characteristics of cells of the macrophage lineage[24].

Assuming that the peptides used in this study can augment phagocytosis by means of an opsonic mechanism, the hypothesis can be proposed that there are two

classes of peptides (or ligands); one which binds to hemocyte receptors and influences cell motility by acting as a chemoattractant, and the other which enhances phagocytosis by selectively coating the particles or micro-organisms to be phagocytized. In both cases, the ligand would have to bind to the appropriate receptor in order to be effective. The consequence of the binding is an effect on cell motility, phagocytosis or both. It is known that in vertebrates, the 'receptors' involved in phagocytosis are operationally defined by the fact that they lack a true, high affinity binding-site for a sterically defined ligand, but are able to interact in a preferential way with chemical groups across a relatively broad spectrum of conformations. The interactions are relatively weak, but are stable as they are generally multiple. On the other hand, receptors involved in phagocytosis can show a high affinity when the particles or micro-organisms to be phagocytized are indirectly recognized by an antibody or complement components[25,26].

The finding that some of the peptides we tested intervene in cell motility but not in phagocytosis can be due to various factors. One possibility is that since immunoglobulin and complement components are not present in gastropods[24,27], high affinity ligand-like receptor complexes cannot form. Alternatively, the way in which the receptor interacts with the ligand could be important, bearing in mind that the N- and C-terminal parts of ACTH behave differently[28].

Another conclusion which emerges from this and previous studies is that not only peptides with a complete aminoacid sequence, such as ACTH and β-endorphin, but also peptide fragments of 4 or 5 aminoacids, are able to stimulate or inhibit immune functions. However, the manner in which this influence is expressed is less uniform than with larger molecules, suggesting that not all aminoacids have the same immunological importance. The influence of small peptides on the immune system has been reported both for bacterial (muramyl peptides) and for animal peptides, such as thymic hormones, tuftin and peptides obtained from colostrum or milk[29]. Even if at present our knowledge of these low molecular weight peptides is poor, the study of their biological activity could, without doubt, be of major heuristic value.

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