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Research Article

Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes

E. Ottaviani[a,*], A. Franchini[a], D. Malagoli[a] and S. Genedani[b]

[a]Department of Animal Biology, via Berengario, 14, University of Modena and Reggio Emilia, I-41100 Modena (Italy), Fax + 39 59 226769, e-mail: ottaviani.enzo@unimo.it
[b]Department of Biomedical Sciences, Section of Pharmacology, University of Modena and Reggio Emilia, I-41100 Modena (Italy)

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Abstract. We report the presence of interleukin (IL)-8-immunoreactive molecules in hemocytes from the mollusc *Mytilus galloprovincialis*. Functional studies demonstrate that recombinant human (rh)IL-8 provokes conformational changes, induces chemotaxis, and increases bacterial phagocytic activity in hemocytes. rhIL-8 induces cell shape changes via protein kinase A and C pathways. These morphological changes are followed by reorganization of the actin microfilaments. The findings suggest that, as previously reported for other cytokines, IL-8 is well conserved and deeply involved in immune functions from invertebrates to mammals.

Key words. Mollusc; hemocyte; immune response; signalling pathway; interleukin-8.

Introduction

Interleukin (IL)-8 and related chemotactic cytokines are small proteins called chemokines that are strongly involved in the mediation of acute inflammation. They are divided into two subfamilies according to the arrangement of the first two cysteines, which are either separated by one amino acid (CXC chemokines) or are adjacent (CC chemokines). In the first family, IL-8 is secreted by various cell types, including monocytes, macrophages, and leukocytes. This chemokine is a potent stimulator of neutrophils, but not monocytes [1, 2]. However, all the information on IL-8 is derived from mammals, while no data are available for invertebrates. In contrast, the presence and functions of other cytokine-like molecules have been described in various tissues of several invertebrates, such as molluscs, insects, annelids, echinoderms, and tunicates [3–10]. IL-1- and IL-6-like molecules have been isolated and characterized in the celomic fluid and celomocytes of the echinoderm starfish *Asterias forbesi*. Starfish IL-1 and IL-6 show characteristics similar to those of mouse and human IL-1, as well as vertebrate IL-6 [11–13]. As far as cytokine functions in invertebrates are concerned, these substances have been demonstrated to elicit cell shape changes and cell migration [3, 14], induce nitric oxide synthase [14], increase phagocytic activity [14], and provoke biogenic amine release [15]. An IL-1-like fraction, designated tunicate IL-1β, has been isolated in hemolymph from the tunicate *Styela clava*, and this fraction is able to stimulate the proliferation of tunicate cells in vitro [16].

To evaluate whether the presence and function of the chemokine IL-8 has been conserved through evolution, we studied hemocytes from the mollusc *Mytilus galloprovincialis*. The modulating effects of recombinant human (rh)IL-8 on immune functions such as cell shape changes, chemotaxis, bacterial phagocytic activity and

* Corresponding author.

the reorganization of the actin microfilaments, and the signalling pathways followed by rhIL-8-induced cellular shape changes have been investigated.

Materials and methods

Hemolymph collection. Specimens of *Mytilus galloprovincialis* Lmk. were collected from rocks in the Adriatic Sea around Cattolica (Rimini, RN, Italy) and maintained in the laboratory for 3 weeks prior to use in the experiments. To avoid bacterial contamination, the artificial sea water was changed twice a week. Hemolymph was collected from the posterior adductor muscle using a 2-ml syringe.

Immunocytochemical assay. The hemocytes were obtained by cytocentrifuging (Cytospin 2 cytocentrifuge, Shandon, UK) the hemolymph on slides at 800 rpm for 3 min followed by air-drying. The immunocytochemical procedure was carried out on unfixed hemocytes using avidin-biotin-peroxidase complex [17], as described in detail elsewhere [18]. Mouse anti-human IL-8 monoclonal antibody (mAb) (1:50) was used as the primary antibody. Negative controls were performed by substituting the primary antibody with non-immune serum or with the primary antibody preabsorbed with the corresponding antigen (in excess) overnight at 4 °C.

In vitro experiments by image analysis of cellular shape changes in the presence of rhIL-8 and signal transduction inhibitors. To test the effects of rhIL-8 on the hemocytes, the hemolymph was incubated with this chemokine at 25, 50, and 100 ng/ml. Marine solution (MS) [19] was used as a control. The specificity of the rhIL-8 effect was evaluated by incubating the hemolymph both with rhIL-8 (100 ng/ml) plus mouse anti-human IL-8 mAb (1:10) and with rhIL-1α (5 pg/ml) plus mouse anti-human IL-8 mAb (1:10). Other experiments were performed after preincubation of the hemolymph for 20 min with either 0.7 mM suramin sodium, 0.5 mM 2′,5′-dideoxyadenosine, 0.6 mM neomycin sulfate, 1.5 mM neomycin sulfate, 0.1 μM calphostin C, 10 μM H-89, or 0.1 μM calphostin C + 10 μM H-89, before adding rhIL-8 (100 ng/ml). The concentrations of the inhibitors are those used in previous studies [20, 21], and have been found to be the most effective without toxic effects in molluscan hemocytes. Moreover, these substances themselves do not alter the shape of hemocytes, giving shape factor (SF) values similar to those observed in the presence of MS alone.

Suramin sodium antagonizes G protein [22], 2′,5′-dideoxyadenosine inhibits adenylate cyclase [23], neomycin sulfate inhibits phosphoinositide metabolism and recycling [24], calphostin C is a specific inhibitor of protein kinase C (PKC) [25, 26], and H-89 is a specific inhibitor of protein kinase A (PKA) [27, 28].

Image analysis. The computer-assisted microscopic image analysis system containing the IA-100™ software (Image Analytics Corporation, Hauppauge, N.Y., USA) was used. One hundred microliters of hemolymph was placed on a microscope slide within a chamber delimited by a vaseline ring, as previously described [19, 29]. Changes in cellular shape from rounded (inactive) to ameboid (active) were detected by measuring the cellular area and perimeter. Cellular shape changes were expressed mathematically using the SF formula of American Innovision (San Diego, CA, USA) analysis system, as described in detail elsewhere [30]. The tested substances were added to the hemocytes when the latter had adhered to the glass. The effects on the cells were recorded after 5 min. SF measurements for each experiment were then taken every 5 min by automatic frame grabbing, following the edges of the cells by hand. All experiments were performed at room temperature.

Chemotaxis assay. The hemolymph was centrifuged at 1700 rpm for 15 min, and the pellet was resuspended in 1 ml of MS containing 0.1% bovine serum albumin (BSA). The chemotactic assays were performed in 48-well microchemotaxis chambers (Nucleopore, Pleasanton, CA, USA), in which the upper and lower compartments were separated by a 5-μm pore, polycarbonate polyvinylpyrrolidone-free filter allowing the cells to migrate actively through the pores. Fifty microliters of cellular suspension was placed in the upper compartment and different concentrations (25, 50, 100 ng/ml) of rhIL-8 in the lower. After 90 min incubation at 37 °C, the migrated cells adhering to the distal part of the filter were fixed, stained, and microscopically identified. Ten fields were counted in each membrane using light microscopy (magnification × 400). Data are expressed as migrated cells/field.

Bacteria. *Aeromonas hydrophila* was grown overnight in tryptic soy broth (Oxoid, Basingstoke, UK) at 37 °C. The bacteria were harvested by centrifugation and suspended in MS. The bacterial concentration was determined using the spread plate method.

In vitro bacterial phagocytosis. Hemolymph was divided into six portions (1 ml) and placed in vials. In three portions (controls), the bacteria *A. hydrophila* (10^6–10^8 bacteria/ml) were added, while the remaining three contained the bacteria plus rhIL-8 at different concentrations (25, 50, and 100 ng/ml). The vials were placed on a revolving mixer for 30 min. The mixtures were then cytocentrifuged onto a slide and stained with toluidine blue. After staining, 200 random hemocytes in each preparation were counted under the light microscope (magnification × 1000) to determine the number of phagocytizing cells. Phagocytosis tests were repeated five times. In each experiment, three slides for each concentration were examined.

Immunofluorescence assay. The effects of rhIL-8 at various times on hemocyte actin microfilaments were studied. Each experiment was repeated three times in duplicate. The hemolymph was divided into 100 µl portions and placed on slides in a ring formed by applying an adhesive PVC strip, in which two 10-mm-diameter holes had been made. For each slide, rhIL-8 at a final concentration of 100 ng/ml was placed in one hole and MS in the other (control sample). Incubation was performed in a humidified chamber for 1, 3, 5, or 10 min. The supernatant was then removed and the adhered hemocytes were air dried, fixed in 4% p-formaldehyde in phosphate-buffered saline (PBS) pH 7.4 (5 min) at 4 °C, washed in PBS, permeabilized with HEPES-Triton X-100 (5 min), incubated in a humidified chamber with fluorescein isothiocyanate (FITC)-labelled phalloidin (10 µg/ml) at 37 °C (30 min), washed again with PBS and mounted in glycerol/PBS. The hemocytes were studied under a Zeiss Axioplan fluorescent microscope, and representative examples were photographed.

Statistical analysis. Statistical analysis was performed by ANOVA, followed by the Student-Newman-Keuls multiple-comparison test.

Chemical reagents. IL-8 was purchased from Pepro Tech, London, UK; recombinant human IL-1α from Genzyme, Boston, MA, USA; anti-IL8 mAb from PharMingen, San Diego, CA, USA; BSA and FITC-labelled phalloidin from Sigma, St. Louis, MO, USA; suramin sodium, 2′,5′-dideoxyadenosine, neomycin sulfate, calphostin C, and H-89 from Biomol Biomolecules, Plymouth Meeting, PA, USA.

Results

Immunocytochemical tests demonstrated that hemocytes of *M. galloprovincials* contain IL-8-like molecules (fig. 1). The immunocytochemical control reactions were always negative (fig. 2).

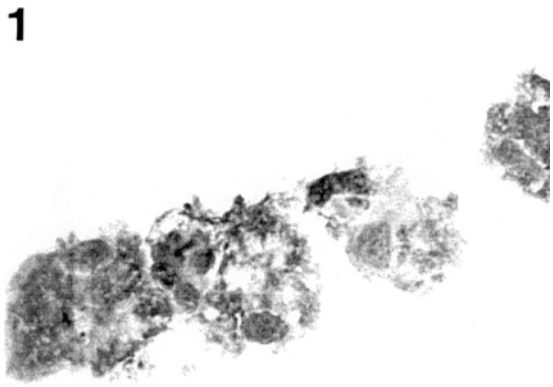


Figure 1. Immunocytochemical staining of *M. galloprovincialis* hemocytes with anti-IL-8 monoclonal antibody. Nuclei were counterstained with hematoxylin. Bar, 10 µm.

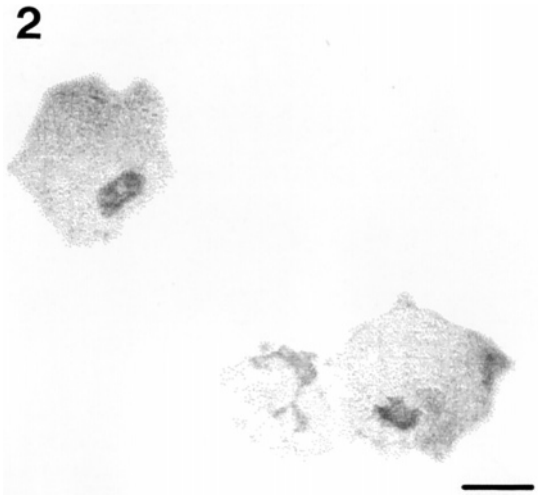


Figure 2. Negative control of immunocytochemical staining of *M. galloprovincialis* hemocytes. Nuclei were counterstained with hematoxylin. Bar, 10 µm.

Computerized microscopic image analysis revealed that rhIL-8 caused changes in cellular shape only at the higher concentration used (table 1, fig. 6). Indeed, at 100 ng/ml, rhIL-8 provoked a significant conformational change in hemocytes ($SF = 0.39 \pm 0.07$) with respect to controls ($SF = 0.77 \pm 0.06$), while at the lower concentrations, the SF values were in the same range as controls. No effect was observed when the hemolymph was incubated with rhIL-8 plus anti-IL-8 mAb, while cell shape changes were seen with rhIL-1α plus anti-IL-8 mAb (data not shown).

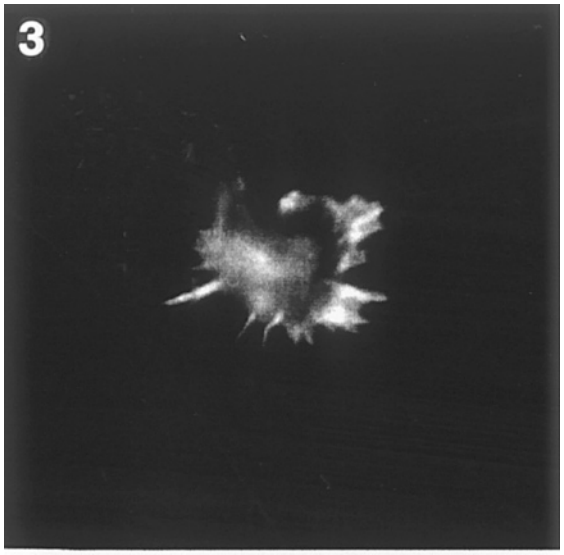
In the chemotaxis experiments, rhIL-8 induced cell migration at the lowest concentrations used (25 and 50 ng/ml; table 2). All three rhIL-8 concentrations provoked a significant increase in bacterial phagocytic activity (table 3).

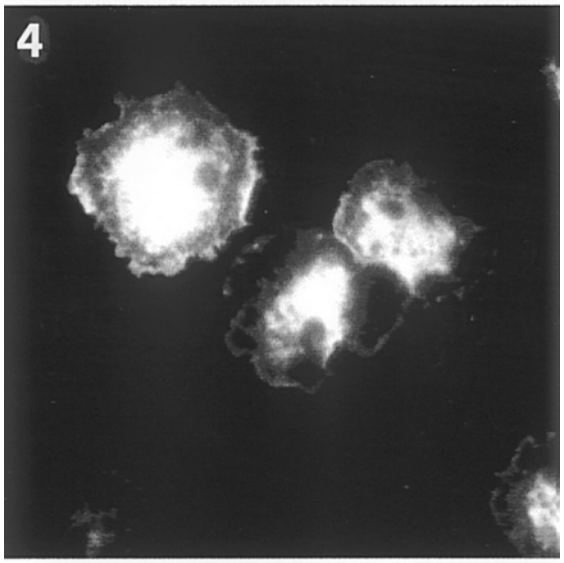
Immunofluorescence studies in hemocytes revealed a time dependence in the pattern of the actin microfila-

Table 1. Hemocyte shape factor (SF) in *M. galloprovincialis* following incubation with rhIL-8 at different concentrations.

Control		0.77 ± 0.06
rhIL-8	25 ng/ml	0.78 ± 0.07
rhIL-8	50 ng/ml	0.75 ± 0.04
rhIL-8	100 ng/ml	0.39 ± 0.07*

The mean $\pm$ SD of ten experiments is shown. Statistical analysis was performed by ANOVA, followed by a multiple-comparison test. * $p < 0.05$ vs control.





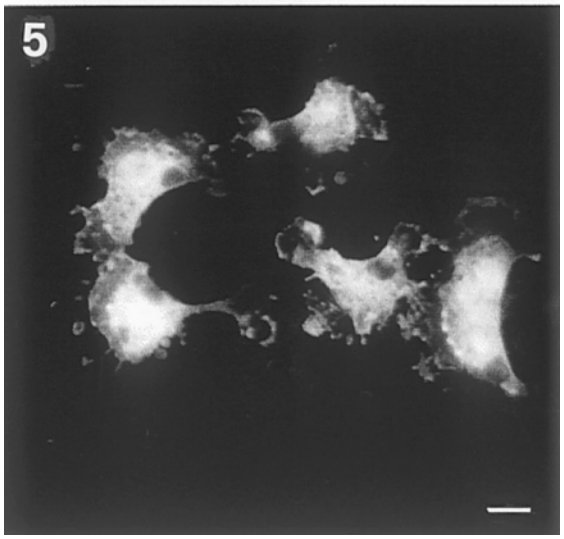


Table 2. Hemocyte chemotaxis in *M. galloprovincialis* following incubation with rhIL-8 at different concentrations.

Control		12.16 ± 5.11
rhIL-8	25 ng/ml	27.10 ± 5.03*
rhIL-8	50 ng/ml	39.60 ± 7.71*
rhIL-8	100 ng/ml	18.53 ± 14.64

The mean $\pm$ SD of the number of hemocytes that migrated through the filter in ten experiments is shown. Statistical analysis was performed by ANOVA, followed by a multiple-comparison test. * $p < 0.05$ vs control.

Table 3. In vitro phagocytosis in *M. galloprovincialis* following incubation with rhIL-8 at different concentrations.

Control		30.57 ± 2.46
rhIL-8	25 ng/ml	40.23 ± 2.69*
rhIL-8	50 ng/ml	41.78 ± 2.55*
rhIL-8	100 ng/ml	57.03 ± 4.41*

The mean $\pm$ SD of the percentage of phagocytic hemocytes in ten experiments is shown. Statistical analysis was performed by ANOVA, followed by a multiple-comparison test. * $p < 0.05$ vs control.

ments since, when adhering to the glass, this cell type changes its morphology. At the beginning, the cells showed phalloidin positivity localized at the cell periphery and, in particular, distributed in thin, radially expanded pseudopods (fig. 3). Three to five minutes after glass adhesion, the cells began to withdraw the pseudopodia and to change shape, assuming a round form. During cell adhesion, the phalloidin positivity was concentrated at the periphery under the plasma membrane (fig. 4). After treatment of the hemocytes with 100 ng/ml rhIL-8, conformational changes were observed, as cells modified from a round to a polarized form. The maximum modification was detected after 5 min treatment and was maintained for the rest of the experiment. The cell shape changes were related to a redistribution of actin. Phalloidin positivity was not uniformly distributed at the periphery, but concentrated in areas where the cells contacted the substrate (fig. 5).

Preincubation of the hemolymph with different inhibitors limited cellular changes (table 4, fig. 6). In particular, the inhibitors of the G protein (suramin sodium) and adenylate cyclase (2′,5′-dideoxyadenosine), and the simultaneous addition to the hemolymph of the

Figures 3–5. Fluorescence micrographs of actin microfilaments of *M. galloprovincialis* hemocytes. Control hemocytes stained with FITC-phalloidin: initially, the positivity is localized both at the cell periphery and in pseudopods (fig. 3); subsequently, the cells assume a round form due to cell adhesion, and the phalloidin positivity is present only at the periphery of the plasma membrane (fig. 4). Hemocytes stained with FITC-phalloidin after preincubation with 100 ng/ml rhIL-8 show a polarized form and phalloidin positivity limited to a few peripheral areas where the cells contact the substrate (fig. 5). It should be noted that the positivity around the nucleus is aspecific, being due to the presence of autofluorescent pigments, e.g., lipofuscins [31]. Bar, 10 μm.

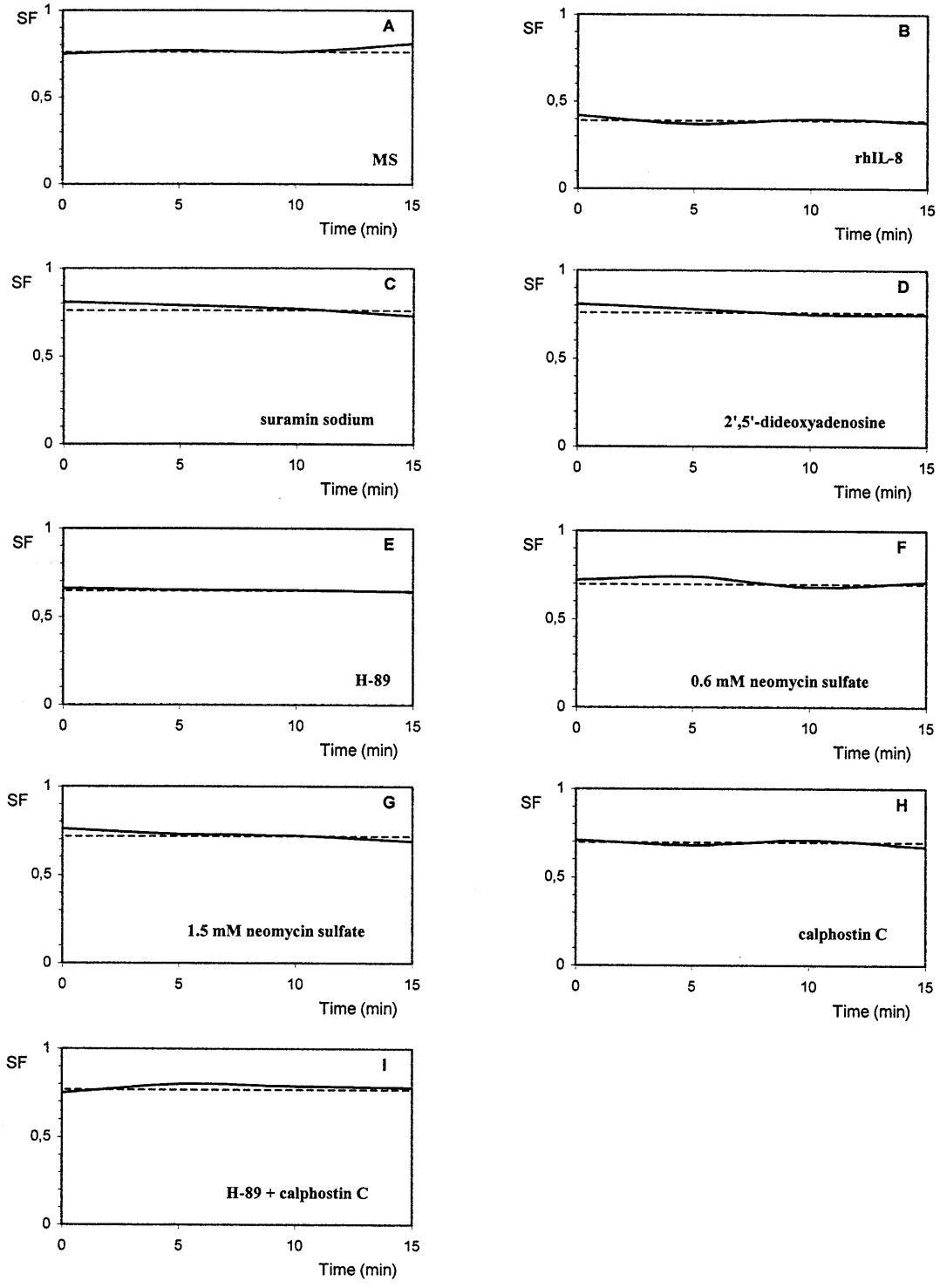


Figure 6A–H. Determination of the hemocyte shape factor (SF) in *M. galloprovincialis* using computer-assisted image analysis following addition to the hemolymph of the single substances MS (*A*) or 100 ng/ml rhIL-8 (*B*), or after preincubation for 20 min with the inhibitors, suramin sodium (0.7 mM) (*C*), 2′,5′-dideoxyadenosine (0.5 mM) (*D*), H-89 (10 µl) (*E*), neomycin sulfate at 0.6 mM (*F*) or 1.5 mM (*G*), calphostin C (0.1 µM) (*H*), and H-89 (10 µM) + calphostin C (0.1 µM) (*I*), followed by the addition of 100 ng/ml rhIL-8. The graphs show the variation in the average SF value recorded at 5-min intervals on five cells. Cell shape was assessed 5 min (time 0) after addition of the substance under investigation. One experiment representative of a set of ten is shown. Statistical analysis was performed by ANOVA, followed by a multiple-comparison test. Bold curve, SF values; dashed line, mean SF value.

PKA (H-89) and PKC (calphostin C) inhibitors, completely blocked the rhIL-8 effect, producing SF values very similar to controls. Neomycin sulfate used at two concentrations to inhibit phosphoinositide metabolism and recycling, and H-89 and calphostin C, tested singly, provoked a partial, although significant, inhibition of the chemokine effect (table 4, fig. 6).

Discussion

One cell type has been described [31] in the hemolymph of the mollusc *M. galloprovincialis*. This hemocyte shows several characteristics that suggest that it might belong to the macrophage lineage. It can adhere to and spread on glass, phagocytize, and express CD11b- and CD16-like molecules on the plasma membrane. Here we report, for the first time, the presence of molecules immunoreactive to IL-8 in invertebrate hemocytes. This result is in agreement with previous studies on other cytokine-like molecules in various invertebrate species [32]. Furthermore, these hemocytes contain growth factor-like molecules, such as platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β1 [18]. We have also demonstrated that cytokines and growth factors play an important role in the immune and neuroendocrine responses in invertebrates [32–35]. rhIL-8 provokes changes in cell morphology, induces cell migration, and increases phagocytic activity in hemocytes. In mammals, IL-8 is known to be involved in inflammation, exerting a potent chemotactic effect on neutrophils, but not on monocytes, suggesting that the molecule could deliver an exact signal to a specific cell type [2]. A different situation is present in our invertebrate model, in which only one cell type is present with characteristics closer to macrophages than to neutrophils. However, this hemocyte is known to play a fundamental role in inflammation reactions in molluscs and other invertebrates [36]. This could explain not only the presence of IL-8-immunoreactive molecules, but also the effects that exogenous rhIL-8 exerts on molluscan hemocytes, indicating that the chemokine may be crucial to basic cell functions, and that these phenomena have been conserved during evolution. Moreover, as reported for other cytokines, such as IL-1α, IL-2 and tumor necrosis factor-α, and for growth factors (PDGF-AB and TGF-β1), rhIL-8 chemotaxis behavior is also dose related [14, 33]. rhIL-8-induced cell shape changes alter the modifications in chemotaxis, as only the higher concentration of rhIL-8 affected hemocytes. Studies performed on human polymorphonuclear leukocytes with other chemoattractants such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) have suggested that different mechanisms underlie the induction of non-directional movement and chemotaxis [37].

Overall, our findings indicate that IL-8 could play an important role in inflammation and host defense in invertebrates. However, rhIL-8 seems to act through two distinct mechanisms: the chemotactic properties, i.e., the expression of cell migration [38], are found at low concentrations, while the cell shape changes, i.e., the expression of cell motility [38], occur at higher concentrations. In contrast, the final step in the immune response, i.e., phagocytosis, is affected at all the concentrations tested, suggesting a prevalent role of rhIL-8 in phagocytic activity rather than in cell shape changes and chemotaxis. In invertebrate host defenses, a dominant role for cytokines has been suggested, and invertebrate IL-1 has been shown to be more potent that vertebrate IL-1 in increasing phagocytic activity [39].

A correlation between cell shape and the amount and distribution of cellular actin has been identified in human neutrophils [40]. Our experiments provide evidence that rhIL-8 provokes morphological and structural modifications in invertebrate hemocytes, reflecting the fact that the contractile cytoskeleton is related to cell motility. The IL-8-induced reorganization of the actin network observed in human neutrophils occurs within 30–60 s [41], whereas the effect of fMLP is more prolonged [42], and a response time of 3–4 min has been reported, comparable with that found in our invertebrate model.

Results obtained using different signalling pathway inhibitors indicate that rhIL-8-induced cell shape changes involve both cyclic AMP and phosphoinositide pathways. The chemokine action was partially inhibited by H-89, a specific inhibitor of PKA, and by calphostin C, a specific PKC inhibitor. However, suramin sodium, which is able to uncouple G proteins

Table 4. Hemocyte shape factor (SF) in *M. galloprovincialis* following incubation with 100 ng/ml rhIL-8 after preincubation of the hemolymph for 20 min with the different inhibitors

Substances	SF
MS	0.77 ± 0.06
rhIL-8	0.39 ± 0.06*
Suramin sodium (1 mg/ml) + rhIL-8	0.77 ± 0.06
2′,5′-dideoxyadenosine (0.5 mM) + rhIL-8	0.77 ± 0.09
H-89 (10 μM) + rhIL-8	0.65 ± 0.10*
Neomycin sulfate (0.6 mM) + rhIL-8	0.70 ± 0.13*
Neomycin sulfate (1.5 mM) + rhIL-8	0.72 ± 0.12*
Calphostin C (0. 1 μM) + rhIL-8	0.69 ± 0.11*
H-89 + calphostin C + rhIL-8	0.78 ± 0.05

The mean ± SD of ten experiments is shown. Statistical analysis was performed by ANOVA, followed by a multiple-comparison test. * $p < 0.05$ vs control.

from cell surface receptors, and 2′,5′-dideoxyadenosine, which inhibits adenylate cyclase activity had strong inhibitory effects, illustrating the importance of these processes in the rhIL-8 transduction signal. The complete blocking of the chemokine effect by inhibition of adenylate cyclase activity suggests that the PKA signalling pathway could be more important than the PKC route in mediating cell shape changes induced by rhIL-8. This is also in agreement with the partial effects elicited by neomycin sulfate at the two concentrations used. In mammals, and in particular in human and guinea pig neutrophils, the effect of IL-8 on chemotaxis is via the PKC pathway [43, 44]. In addition, the chemotactic function is down-regulated by increased intracellular levels of cAMP [44].

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