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Immunology

Stimulatory effects of opioid neuropeptides on locomotory activity and conformational changes in invertebrate and human immunocytes: Evidence for a subtype of δ receptor

(invertebrate immune reactions/opioid receptors/[D-Ala^2,D-Met^5]enkephalinamide/immunocyte conformational changes)

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ABSTRACT **The presence of opioid neuropeptides was shown to stimulate conformational changes and locomotory activity in immunocytes of two representatives of invertebrates as well as in human leukocytes. Cells were examined by use of phase-contrast and Nomarski optics coupled with a Zeiss Axiophot microscope, and of the Zeiss Videoplan/Vidas Image Analysis system. Immunocompetent blood cells, activated by exogenous opioids or stressful stimuli presumed to engage endogenous opioids, showed flattening, elongation, and formation of pseudopodia. In the mollusc *Mytilus edulis*, ameboid movements resulted in the formation of cell clusters, an activity not observed in untreated controls, or in immunocytes simultaneously exposed to opioid and naloxone. Tests with nine immunoreactive substances revealed immunocyte stimulation by δ-, μ-, κ-, and ε(?)-selective ligands. One of these, [D-Ala^2,D-Met^5]enkephalinamide (DAMA), active at a concentration of 10 pM, proved to be considerably more effective than the rest. The high pharmacological potency of DAMA, observed in both human and invertebrate immunocytes, sets this opioid apart from the closely related [D-Ala^2,D-Leu^5]enkephalin, a discrepancy not occurring in the mammalian nervous system. This suggests a specific function for [Met]enkephalin in immunoregulation, mediated perhaps by a special subtype of δ receptor.**

Numerous recent studies have demonstrated interactions among the nervous, the endocrine, and the immune systems (for reviews, see refs. 1–3). These bidirectional channels make use of the same signal molecules—namely, neuropeptides, serotonin, and lymphokines. In addition, it has been shown that enkephalin-like and other neuropeptides are involved in internal immunoregulatory activities (3).

In a previous study (4), evidence has been presented to indicate that also in invertebrates immunocompetent hemocytes (immunocytes) use opioid neuropeptides in such autoregulatory processes. In the mollusc *Mytilus edulis* and the insect *Leucophaea maderae*, two such processes, cellular adherence and migration, have been shown to be enhanced in the presence of [D-Ala^2,D-Met^5]enkephalinamide (DAMA), a stable synthetic [Met]enkephalin analogue. The operation of stereospecific receptors in this process was demonstrated by the fact that naloxone (10 nM) markedly reduced these effects (4).

An additional result of these *in vitro* studies was the observation that the presence of opioid substances brings about distinctive conformational changes in responsive immunocytes as well as stimulation of their locomotory behavior. These effects of opioids on immunocytes of *Mytilus* and *Leucophaea* were subjected to a detailed Zeiss Videoplan/Vidas Image Analysis coupled to time-lapse video analysis in the present study.

For comparison between invertebrate and mammalian systems, human leukocytes were subjected to some of the same tests.

METHODS

Subtidal *M. edulis* were collected from the shore areas of Wading River and Queens College/Caumsett State Park of Long Island Sound, New York, during the months of November and December 1988 and January 1989 and were maintained in the laboratory for up to 3 weeks prior to testing. They received frequent changes of fresh seawater filtered through gauze. Adult specimens of *L. maderae* were taken from stock colonies maintained in the laboratory for many years. Human blood cells (whole blood), prepared by standard laboratory procedures, were obtained from the Long Island Blood Center (Melville, NY). The Student's *t* test was used for statistical analysis of the results.

For an *in vitro* analysis of immunocyte responses to DAMA and other stimulatory substances (see Table 1) in *Mytilus*, 0.1 ml of hemolymph was withdrawn from the extracellular space in the area of the posterior adductor muscle, mixed by Vortex with either 0.1 ml of Instant Ocean or Instant Ocean plus drug, and smeared on a slide or coverslip precoated with a 2% bovine serum albumin solution, as previously noted (4). Inverted coverslips, placed over a slide on a ring of vaseline (to prevent drying out) permitted time-lapse video recording for periods up to 2 days. The exogenous substances added to the incubation medium, either alone or together with the antagonist naloxone, are listed in Table 1. The mixture was allowed to incubate at room temperature (23°C) for 15 min or longer.

Hemolymph from *Leucophaea* was collected from the thoracic or abdominal region, added to an equal volume of a coagulation inhibiting solution (see ref. 4), and handled in the same way as in *Mytilus*.

The cell preparations were examined by use of phase-contrast and Nomarski optics coupled with a Zeiss Axiophot microscope. Measurements were taken with the Zeiss Videoplan/Vidas Image Analysis system. For this purpose, specific images were converted to binary images following frame grabbing. To speed up the process of data acquisition, we developed an image analysis procedure macrocustomized for this project. Simultaneously, specific cells were photographed by an internal Zeiss Automated Program Photography system (35-mm film) along with video photography with a time-lapse video synchronization system (JVC). Observations were made immediately, as well as at 5-min intervals

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Abbreviations: DAMA, [D-Ala^2,D-Met^5]enkephalinamide; DADLE, [D-Ala^2,D-Leu^5]enkephalin.

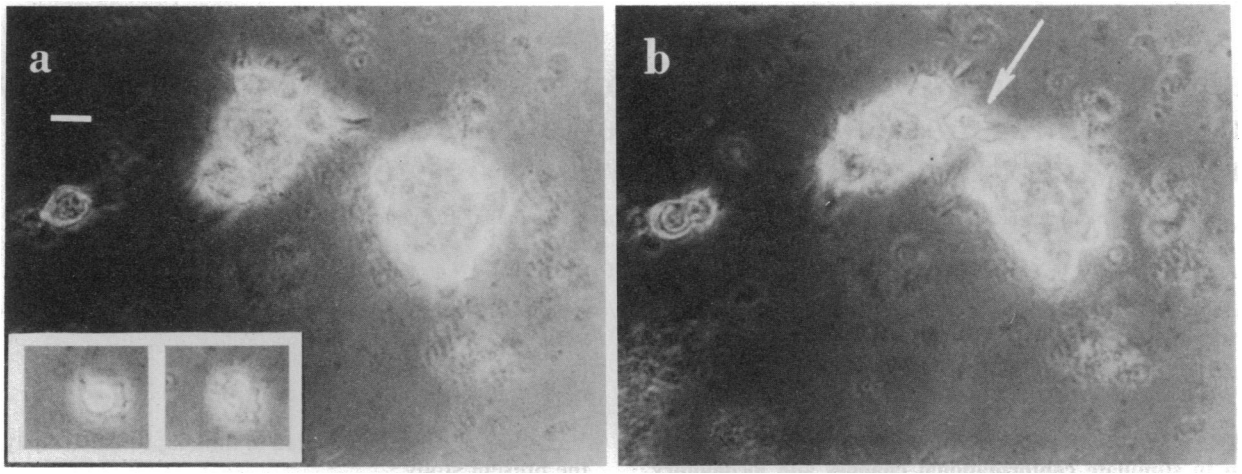

FIG. 1. Photomicrographs of *Mytilus* immunocytes, based on video observation, showing effect of exposure to DAMA (10 pM). (*Left Inset*) Cell prior to stimulation by drug (area, 141 μm^2). (*Right Inset*) Same cell 15 min after administration of drug (increase of area to 189 μm^2). (*a* and *b*) Formation of cell clusters approaching each other and attracting additional cells, recorded at intervals of 18 and 20 min after exposure to DAMA. (*a*) Note cell in left cluster with pseudopodia directed toward second cluster at right. (*b*) Same cell having made contact with second cluster (arrow). (Bar = 10 μm.)

thereafter. The locomotory behavior of individual immunocytes was recorded in the presence or absence of drugs by direct observation and videotapes.

Changes in cellular conformation from inactive-rounded to active-ameboid, based on measurements of cellular area and perimeter, were mathematically expressed by use of the form-factor-pe calculation of the Zeiss Vidas Analysis system, whereby the equation $(4 \times \pi \times \text{area})/\text{perimeter}^2$ provides mean numerical values. The lower this number, the higher the cellular perimeter and the more ameboid the cellular shape.

For tests examining the effect of stress on immune responses in *Mytilus*, groups of five animals each were placed in fresh seawater at room temperature. One group (controls) was allowed to remain undisturbed. Other groups received various "stressful" treatments. Neither successive brief electrical shocks of 1 V for 10 ms on the in-current siphon in themselves, nor the presence of a wedge preventing closure of the siphon, caused enough of a disturbance to alter the behavior of the animals' hemocytes or their capacity to respond to DAMA and naloxone. However, the combination of both treatments proved to be effective as judged by its effect on the animal's defense system.

RESULTS

In slide tests with hemolymph of *Mytilus* and *Leucophaea*, prepared as described and viewed with Nomarski optics, the granulocytes, presumed to be immunocompetent (5), appear for the most part either spherical or ovoid (Figs. 1 and 2). In control preparations (absence of drugs) of *Mytilus* hemocytes the cellular areas measure approximately 55–200 μm^2. In the process of changing from adhering to mobile cells showing mild random movements, some of the hemocytes (5–10%) increase in size by ≈25%.

The addition of specific drugs to the hemolymph preparations appears to evoke immediate responses in ≈35% ($P > 0.01$) of the total cell population. In particular, the presence of DAMA (10 pM) markedly stimulates the locomotory activity of these cells, which becomes most noticeable within a 15- to 30-min period (Fig. 1). At the outset, activated cells move randomly and then they gradually form aggregations of increasing size, an activity not observed in untreated hemolymph. The conformational changes these hemocytes undergo before becoming mobile include flattening (Fig. 1*a Inset*) and the formation of pseudopodia showing cytoplasmic streaming (Fig. 1). Cellular areas of activated cells exhibiting ameboid movements may increase by as much as 65% (extreme case; Figs. 1 and 3*A*). Distinctive granular accumulations become more noticeable in the cytoplasm than in unstimulated cells. The numerical value expressing the relative increase in cellular perimeter, obtained by the form-factor-pe calculation was 0.37, as compared with that of 0.78 for unstimulated control cells (Fig. 3*C*). Pseudopodia of ameboid hemocytes seem to be oriented preferentially in the direction of cell clusters of the same type (Fig. 1). Careful

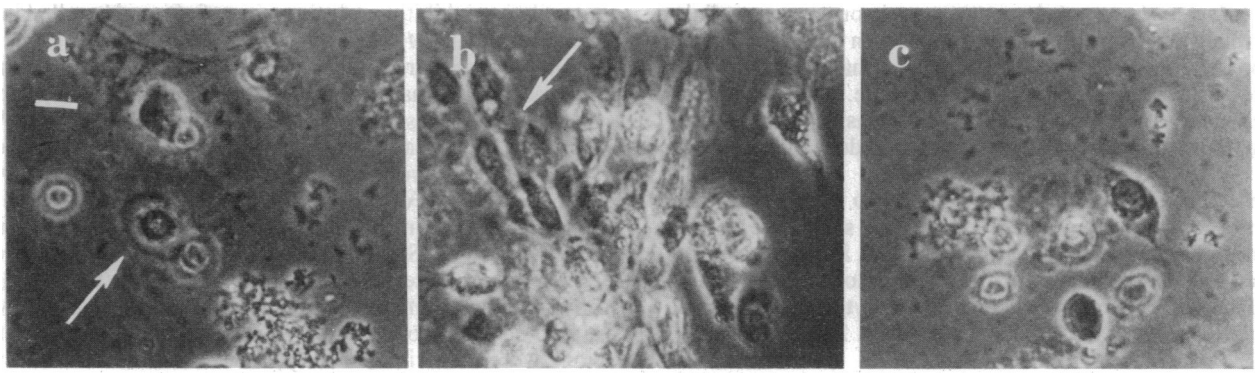

FIG. 2. Immunocytes of *Leucophaea*. (*a*) Prior to opioid stimulation; note rounded cells (arrow). (*b*) Thirty minutes after exposure to DAMA (10 pM); note elongated activated cells (arrow). (*c*) Thirty minutes after exposure to both DAMA and naloxone (10 nM); note blocking activation of immunocytes. Means of form-factor-pe values for *a*, *b*, and *c* are 0.81, 0.47, and 0.79, respectively. (Bar = 10 μm.)

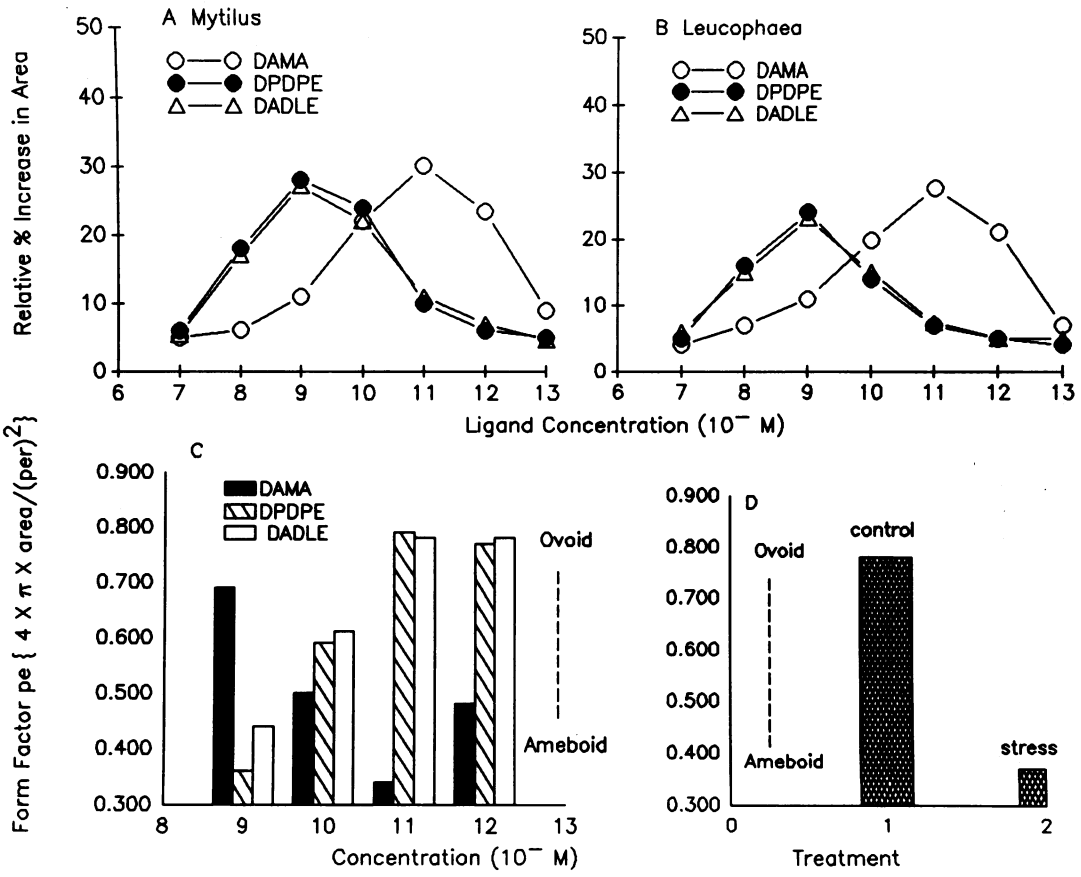

FIG. 3. (*A–C*) Effects of three δ-selective opioid ligands on immunocyte structure. Increase in cellular area in *Mytilus* (*A*) and *Leucophaea* (*B*). (*C*) Change in form-factor-pe values expressing cellular shape in *Mytilus*. (*D*) Difference between form-factor-pe values of cells from normal control and stressed (electrical stimulation coupled with the wedge as noted in the text) *Mytilus*. DPDPE, [D-Pen^2,D-Pen^5]enkephalin (Pen, penicillamine).

observation of cellular movements on the video screen reveals that, at comparable distances, larger cell clusters tend to attract more wandering cells than smaller accumulations.

The response of immunocytes of *Leucophaea* to the same experimental conditions generally parallels that in *Mytilus* but shows certain characteristics (Figs. 2 and 3*B*). One of these is the elongate appearance of stimulated hemocytes. Another difference is that their activation by DAMA does not elicit the same degree of locomotory activity as in *Mytilus*, probably because of the high viscosity of the hemolymph fluid of this insect.

An important feature observed in both representatives of invertebrates examined is that the addition of naloxone, at a concentration of 10 nM prior to or concurrent with the cells' exposure to exogenous opioids, blocks their activation (Fig. 2*c*). It has to be pointed out, however, that at higher concentrations naloxone, like opioids, can enhance conformational as well as locomotory phenomena.

Another item of interest addressed in this study was the comparison of the stimulatory effects of DAMA on cellular conformation and locomotory activity with those observed in the presence of related neuropeptides or nonpeptide substances (Table 1; Fig. 3). Eight additional drugs tested yielded cellular responses comparable to those observed with DAMA, but all of these proved to be less potent.

The hemolymph of stressed *Mytilus* showed a considerably higher number of hemocytes than that of unstressed, indicating their mobilization from various tissues of the organism (Fig. 4; see also ref. 5). Moreover, the proportion of activated—i.e., polymorphic—ameboid cells by far exceeded that in untreated specimens (Fig. 3*D*). The degree of conformational changes brought about by the stressful stimuli administered, expressed by the value of 0.35, indicates an increase in cellular perimeter that is virtually the same as that in opioid-activated nonstressed specimens (referred to above).

Table 1. Opioid and nonopioid substances stimulating conformational and locomotory changes in invertebrate and human immunocytes grouped according to receptor selectivity of ligands

Ligand	Effective concentration, nM	
	Cell perimeter change	Cell area change
Invertebrate		
δ		
DAMA	0.01	0.01
DADLE	1.0	1.0
DPDPE	1.0	1.0
μ		
DAGO	1.0	1.0
β-Casomorphin	1.0	1.0
κ		
Dynorphin	1.0	1.0
Bremazocine*	1.0	10.0
Ethylketocyclazocine*	10.0	10.0
ε (?)		
β-Endorphin	0.1	0.1
Human		
DAMA	0.01	0.01
DADLE	10.0	1.0

DADLE, [D-Ala^2,D-Leu^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; β-casomorphan, H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH.
*Nonopioid substances.

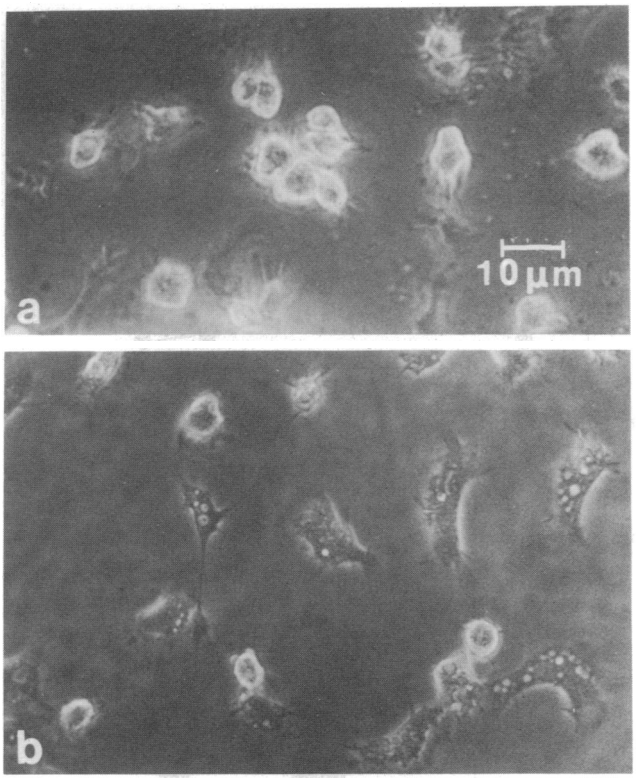

FIG. 4. Immunocytes from control (*a*) and stressed (*b*) *Mytilus*. In *b*, note activated ameboid cells with cytoplasmic granules. Means of form-factor-pe values: 0.80 (*a*) and 0.39 (*b*).

It is of interest that the conformation of activated hemocytes of stressed *Mytilus* did not change further in response to an additional challenge—i.e., the administration of opioid drugs. Moreover, the proportion of inactive rounded versus ameboid cells in the stressed specimens remained about unchanged after the addition of DAMA, suggesting that the unaffected cells represent a hemocyte population that is unresponsive to immunostimulation. The administration of naloxone (10 nM) to animals prior to that of stressful stimuli yielded hemolymph preparations with cellular components, very few of which appeared to have become activated. By contrast, once activated as a consequence of induced stress, immunocytes no longer responded to the administration of this opioid antagonist—i.e., ameboid cells did not return to the rounded inactive form. This indicates that, in unstressed opioid-activated as well as stressed-activated immunocytes, naloxone seems to be able to block the process of mobilization, but once it has occurred it cannot reverse it.

In vitro tests with human blood cells revealed the same kind of response to DAMA, at a concentration of 10 pM, as that of the immunocytes of the invertebrate species examined—namely, stimulation of locomotory behavior and changes in cellular morphology (Fig. 5). In the responsive population of leukocytes (≈28% of the total number of cells viewed) DAMA brought about an increase in cellular area amounting to 21–33%. In 19 readings, the treated cells measured from 179 to 235 (mean, 208) μm^2, as compared to values in untreated control cells (21 readings) in the range of 142 to 156 (mean, 149) μm^2. Most of the responsive cells showed signs of activation within 5 min of the application of DAMA. The activated human immunocytes (Fig. 5) resemble those of *Mytilus* and *Leucophaea*, but they appear less ameboid than those of *Mytilus*. The form-factor-pe value of DAMA-treated human leukocytes ranged from 0.67 to 0.20 (extreme case) as compared to the control value of 0.89. Cells exposed to naloxone (10 nM), either before or simultaneously with DAMA (1 µM to 0.1 pM), were unresponsive to the opioid.

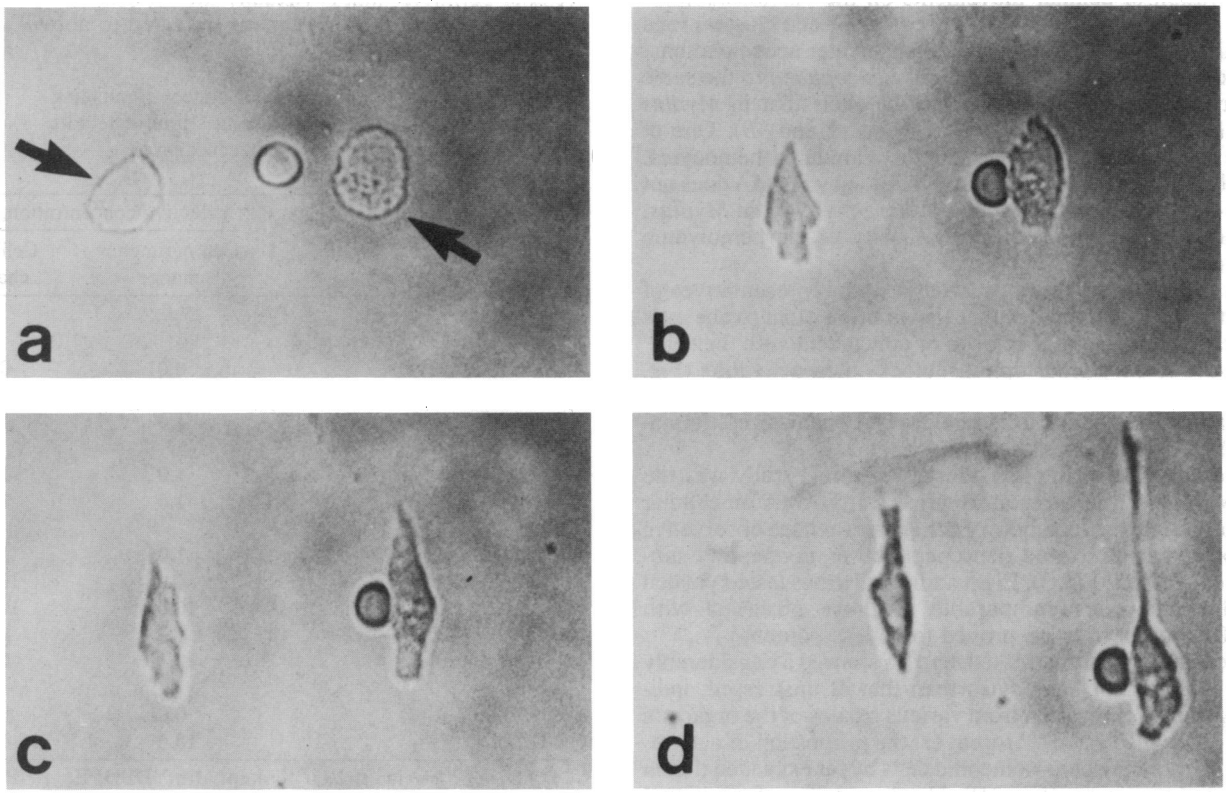

FIG. 5. Stimulation of human leukocytes by DAMA (10 pM). (*a*) Unstimulated cells (arrows) next to an erythrocyte. (*b–d*) Same cells exposed to opioid observed at intervals of 1.5, 3, and 4.5 min. Note gradual elongation. Means of form-factor-pe values: 0.81 (*a*) and 0.41 (*d*).

[D-Ala^2,D-Leu^5]Enkephalin (DADLE) also influenced the conformational changes in these immunocytes, but the effective concentrations of this drug (cell perimeter, 10 nM; cellular area, 1 nM) were not as low as that of DAMA. In the responses to both opioids the morphological alterations occurred prior to the stimulation of locomotory activity. This is in contrast to the report of Falke and Fischer (6), who failed to induce conformational changes by DADLE in mammalian polymorphonuclear leukocytes.

DISCUSSION

In counteracting antigenic challenges and other stress-related phenomena, invertebrate as well as vertebrate animals make use of neuropeptides and additional messenger substances released by neuroendocrine centers as well as immunoregulatory cells. One manifestation of stimulation of invertebrate immunocytes by opioids, analyzed in some detail in the present study, is the enhancement of their locomotory activity accompanied by characteristic changes in cellular structure. These conformational changes are comparable to those observed after exposure of immunocytes to opioid and certain nonopioid substances observed in mammalian systems by Falke and Fischer (6).

In *Mytilus*, the enhancement of chemokinetic activity by exogenous opioids was followed by the formation of immunocyte clusters, indicating that single ameboid cells are chemotactically attracted by cells of the same kind. The possibility that this temporally distinct, oriented locomotory activity may also be influenced by opioids is suggested by an earlier *in vivo* experiment (4) showing directed migration of *Mytilus* immunocytes to the site of injection of DAMA. The fact that this response was not merely due to the nonspecific irritation of the puncture was established by control injections without DAMA. This clearly indicates the capacity of these cells to be guided by spatial differences in opioid concentration. However, the operation of a second messenger substance giving direction to randomly moving opioid-stimulated immunocytes cannot be ruled out. The demonstration of such an activity could explain satisfactorily why, under the conditions of the *in vitro* experiments—i.e., the presumed absence of an effective concentration gradient of exogenous opioid—the presence and relative size of a cell cluster should influence the process of aggregation in the manner observed.

The results obtained in stressed *Mytilus* are a contribution to the large body of information on the diverse effects of stressful stimuli on the mammalian immune system (e.g., see ref. 7). The fact that naloxone prevents stress-induced immunomodulation in this invertebrate indicates that endogenous opioids are involved in this process. It is of further interest that the immunocytes, after having become "fully activated," either in the presence of exogenous opioid or under stressful conditions, no longer respond to the drug or its antagonist. This demonstrates that the respective receptor mechanism is subject to varying physiological conditions.

Another issue to be discussed concerns the occurrence in immunoactive cells of multiple receptors, each presumed to respond more or less selectively to a specific type of neuropeptide with a distinctive functional role. Whereas heterogeneity of receptor subtypes in the mammalian immune system is fairly well established (1–3), no corresponding information on this problem in invertebrates has been available thus far. What the present study has added to this picture is summarized in Table 1. Most of the substances tested, including δ-, μ-, and κ-selective ligands, elicited responses at concentrations no lower than 1 nM. The potency of the putative ε-selective opioid β-endorphin was 0.1 nM.

DAMA was the only ligand that was most effective at a concentration as low as 10 pM. It is of particular significance that DAMA proved to be equally potent in the tests with human leukocytes. The distinctly lower effectiveness of the closely related opioid DADLE in both human and invertebrate immune reactions is in contrast to the "classical" situation in the mammalian nervous system, where this discrepancy in binding potency does not exist (8). A result related to these data is the high potency of [Met^5]enkephalin (1 pM) observed in immunoregulatory granulocyte–endothelial cell interactions demonstrated in human umbilical veins (9).

The presence of an endogenous [Met]enkephalin-like material demonstrated in *Mytilus* immunocytes (4) and the high degree of specificity of DAMA shown in the present study suggest a distinctive role for [Met]enkephalin in immunoregulatory processes and the possible existence of a special subtype of δ receptor responsive to this ligand. Even though detailed information on these phenomena is still scanty, their further exploration on a comparative basis promises to prove useful in a variety of mammalian studies, including those concerned with immune responses to stress and other neuroendocrine conditions, receptor specificity, and pharmacological testing.

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1. Sibinga, N. E. S. & Goldstein, A. (1988) *Annu. Rev. Immunol.* **6**, 219–249.
2. Bost, K. K. (1988) *Allergy* **43**, 68–83.
3. Blalock, J. E. (1989) *Physiol. Rev.* **69**, 1–32.
4. Stefano, G. B., Leung, M. K., Zhao, X. & Scharrer, B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 626–630.
5. Stefano, G. B., Zhao, X., Bailey, D., Metlay, M. & Leung, M. K. (1989) *J. Neuroimmunol.* **21(1)**, 67–74.
6. Falke, N. E. & Fischer, E. G. (1985) *Immunobiology* **169**, 532–539.
7. Plotnikoff, N. P., Faith, R. E., Murgo, A. J. & Good, R. A. (1986) *Enkephalins and Endorphins Stress and the Immune System* (Plenum, New York).
8. Itzhak, Y. (1988) in *The Opiate Receptors*, ed. Pasternak, G. W. (Humana, Clifton, NJ), pp. 95–142.
9. Boogaerts, M. A., Vermylen, J., Deckmyn, H., Roelant, C., Verwigghen, R. L., Jacob, H. S. & Moldow, C. F. (1983) *Thromb. Haemostasis* **50**, 572–575.