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Immunology

Opiate-like substances in an invertebrate, an opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression

(morphine/invertebrate ganglia/human monocytes/chemotaxis/*Mytilus edulis*)

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ABSTRACT **The presence of morphine-like and codeine-like substances was demonstrated in the pedal ganglia, hemolymph, and mantle tissues of the mollusc *Mytilus edulis*. The pharmacological activities of the endogenous morphine-like material resemble those of authentic morphine. Both substances were found to counteract, in a dose-dependent manner, the stimulatory effect of tumor necrosis factor α or interleukin 1α on human monocytes and *Mytilus* immunocytes, when added simultaneously to the incubation medium. The immunosuppressive effect of this opiate material expresses itself in a lowering of chemotactic activity, cellular velocity, and adherence. Codeine mimics the activity of authentic morphine, but only at much higher concentrations. Specific high-affinity receptor sites (μ_3) for morphine have been identified on human monocytes and *Mytilus* immunocytes. In *Mytilus* recovering from experimentally induced stress, the return of "alerted" immunocytes to a more inactive state appears to be due to a significant rise in the content of morphine-like material in the pedal ganglia and hemolymph at this time. Thus, morphine may have a role in calming or terminating the state of immune alertness.**

The occurrence throughout the animal kingdom of messenger molecules identical with, or closely related to, those known in mammals has been amply documented by biochemical identification, functional analysis, and the demonstration of specific receptor sites (1–6). This applies in particular to classical neurotransmitters, neuropeptides, and cytokines. The demonstration by Spector and coworkers (9–11) and others (7, 8) of endogenous morphine and of opiate receptors has thus far been restricted to vertebrates. The present report examines the presence of both morphine-like and codeine-like substances in the nervous system and hemolymph of an invertebrate, the mussel *Mytilus edulis*, and identifies a specific opiate receptor in immunoactive cells.

MATERIAL AND METHODS

Biochemical Demonstration of Morphine and Codeine. Pedal ganglia, mantle, and hemolymph of subtidal *Mytilus edulis*, collected from the shore area of Montauk Point, Long Island Sound, NY, were separated immediately upon arrival in the laboratory and stored at −70°C for the determination of opiate alkaloids.

Extraction method 1. Stored tissue samples were thawed and homogenized (Polytron) in 10 vol of 0.01 M HCl [12 M, added to the sample in a 6:10 (vol/vol) ratio (HCl/sample) to a final strength of 10%]. The samples were then heated at 100°C for 30 min, cooled, and centrifuged at 10,000 × *g* for 20 min. The resulting precipitate was discarded and the supernatant was extracted with 5 vol of 10% (vol/vol) 1-butanol in chloroform. The supernatant was adjusted to pH 8.8–9.0. The organic phase was back-extracted into 2 vol of 0.01 M HCl. Morphine recovery was 50–70%. The aqueous phase was evaporated to dryness and dissolved in 10 ml of phosphate-buffered saline (PBS, pH 7.4) and then the pH was adjusted to 8.5–9.0. Samples were passed through a Sep-Pak C_{18} cartridge. Morphine and codeine were eluted from the cartridge with 7 ml of 0.1 M pyridine in acetic acid (pH 6.2) containing 2.5% (vol/vol) 1-propanol. The eluate was evaporated to dryness and dissolved in 250 μl of 1 mM HCl. An aliquot of 200 μl was injected onto a C_{18} reverse-phase HPLC column (LiChrosorb, 0.4 × 25 cm, Merck). The samples were eluted with 0.1 M pyridine in acetic acid (pH 6.2) followed by a linear gradient of 0–25% 1-propanol/0.1 M pyridine in acetic acid at a flow rate of 1.5 ml/min. Four 1-min fractions were collected during morphine and codeine elution as determined by known standards. These fractions were then evaporated, dissolved, and assayed for morphine and codeine by a sensitive radioimmunoassay (RIA) described by Spector and coworkers (12–15). Polyclonal antibodies generated against 3-carboxymethylmorphine and *N*-carboxymethylmorphine-bovine serum albumin were used to monitor morphine and codeine immunoreactivity. ^{125}I-labeled morphine (Hoffmann–LaRoche) was used as a tracer. The detection limit of this assay is 15 fmol per tube. Codeine and morphine react equally well (IC_{50}, 0.03 nM) with the polyclonal antibody. Since codeine has an elution pattern on the C_{18} column distinct from that of morphine, it can easily be separated in different eluted fractions and assayed with the same antibody. Under the described experimental conditions, the retention times of morphine and codeine were 5 and 13 min, respectively.

Extraction method 2. Mussel pedal ganglia [1.9 g (wet weight)] were homogenized in 6 ml of 60% (vol/vol) methanol in 2 M acetic acid. All extraction steps were carried out at 4°C. After centrifugation at 15,000 × *g* for 20 min, the supernatant was lyophilized and redissolved in 400 μl of 1 M acetic acid. The extract was clarified by centrifugation and fractionated by HPLC with the use of a Brownlee C_{18} column. Three hundred microliters of the extract was chromatographed at 35°C at a flow rate of 1 ml/min with a binary gradient consisting of 10% (vol/vol) acetonitrile in 20 mM

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Abbreviations: IL-1α, interleukin 1α; TNF-α, tumor necrosis factor α; 3DHM, [^{3}H]dihydromorphine; ff, form factor.

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potassium phosphate (pH 3.0) as solvent A and acetonitrile as solvent B. The run was made with a linear increase of solvent B from 0 to 40% in 20 min, and 0.5-ml fractions were collected. Under these conditions the retention times for morphine and [Met]enkephalin were 15 and 16 min, respectively. Two hundred microliters was removed from fractions 3, 31, and 33 and lyophilized. Fraction 3 was used as a blank; fractions 31 and 33 contained eluants with retention times corresponding to morphine and [Met]enkephalin. Each sample was dissolved in 100 μl of PBS and bioassayed for morphine and [Met]enkephalin with human monocytes as described below.

Chemotaxis Assay. Monocytes, obtained from either the Long Island Blood Service (Melville, NY) or the Department of Surgery, University Hospital, State University of New York at Stony Brook, and *Mytilus* immunocytes were processed as reported in detail (16–19) and tested for their response to opiates. Effects on cellular conformation were examined after incubation with various osmotically balanced drugs in combination or alone (morphine, naloxone, HPLC fractions from methods 1 and 2, HPLC fractions plus naloxone, and vehicle alone), and a cover slip was placed on the preparation (16–19). This mixture was incubated at 37°C for 30 min. The numerical values obtained are the mean taken from 10 to 20 readings per slide replicated four times and analyzed by Student's *t* test.

Axis alignment of activated cells demonstrating chemotactic behavior was evaluated as described elsewhere (19) by use of the American Innovision (San Diego) image analysis software (16–19). Monocytes placed in a chemotactic environment containing a concentration gradient of interleukin 1α (IL-1α) or tumor necrosis factor α (TNF-α) align their main axis in the direction of the gradient. For our assay, the monocytes were exposed to IL-1α (10 units/ml) and then to morphine (10 nM) or to morphine (10 nM) and naloxone (10 nM) as well as to fractions of HPLC extracts showing positive morphine, codeine, or enkephalin immunoreaction. Changes in cellular velocity were recorded to provide additional indirect information on the cell's chemotactic capacity.

Opiate Binding Analysis. Human monocytes obtained from the Ficoll/Hypaque centrifugation and subsequent washing were homogenized in 50 vol of 0.32 M sucrose (pH 7.4) at 4°C, by using a Brinkmann Polytron (30 s, setting 5). The crude homogenate was centrifuged at 900 × *g* for 10 min at 4°C, and the supernatant was reserved on ice. The whitish crude pellet was resuspended by homogenization (15 s, setting no. 5) in 30 vol of 0.32 M sucrose/50 mM Tris·HCl, pH 7.4, and centrifuged at 900 × *g* for 10 min. The extraction procedure was repeated one more time, and the combined supernatants were centrifuged at 900 × *g* for 10 min. The resulting supernatants (S1′) were used immediately.

Immediately prior to the binding experiment, the S1′ supernatant was centrifuged at 30,000 × *g* for 15 min, and the resulting pellet (P2) was washed once by centrifugation in 50 vol of the sucrose/Tris·HCl. The P2 pellet was then resuspended with a Dounce hand-held homogenizer (10 strokes) in 100 vol of buffer. Binding analysis was then performed on the cell membrane suspensions.

Mytilus immunocytes were obtained from 1150 animals and prepared for binding and pharmacological analysis as described elsewhere in detail (6, 19).

Stress Experiments. For tests examining the effect of morphine on the stress response of *Mytilus* immunocytes, the animals were subjected, as reported elsewhere in detail (20), to repeated electrical shocks through the surface over the pedal ganglia and a wedge preventing the closing of the valves. An *in vitro* analysis of the conformational changes in hemocytes from stressed animals, as compared with hemocytes in unstressed controls, was carried out.

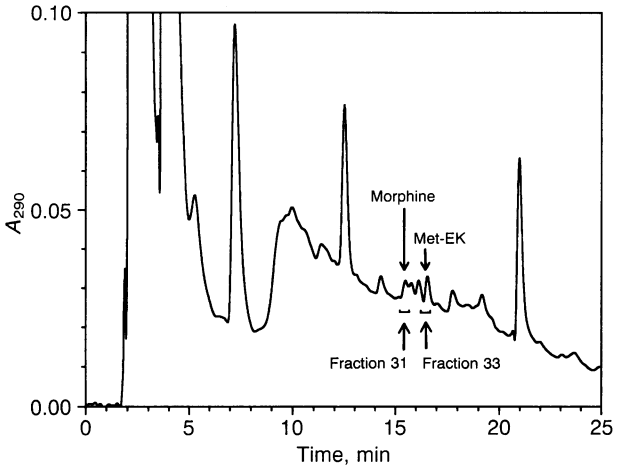

FIG. 1. HPLC chromatogram showing the fractions of the pedal ganglia extract from method 2 used for bioassay of morphine-like and Met-enkephalin-like materials in immunocytes (for bioassay procedure see Table 1). Arrows indicate the migration positions of the morphine and [Met]enkephalin (Met-EK) standards.

[^{3}H]Dihydromorphine (3DHM) was obtained from New England Nuclear. Recombinant TNF-α and IL-1α were obtained from Genzyme. One unit of TNF-α is defined as the amount required to double the proliferative response of mouse thymocytes stimulated with 1 μg of phytohemagglutinin per ml.

RESULTS

Presence of Morphine-Like Material in an Invertebrate. The pedal ganglia of the mollusc *Mytilus edulis* were found to contain morphine-like and codeine-like substances, considered to be endogenous, as documented by two extraction and HPLC procedures. The morphine-like and codeine-like materials from method 1 were verified by RIA and chemotaxis assay; the morphine-like and [Met]enkephalin-like materials from method 2 were verified by chemotaxis assay. The elution times of the morphine-like and [Met]enkephalin-like materials from method 2 correspond exactly to their respective standards (Fig. 1). Hemolymph and some nonnervous tissues (i.e., those of the mantle) also yielded various amounts of this opiate (Fig. 2). However, the material in the mantle could be washed out, indicating that it may accumulate there passively from hemolymph diffusing through the tissues.

Pharmacological Characterization of Endogenous Opiates. The pharmacological activity of the morphine material extracted from *Mytilus* nervous tissue mimicked that of authentic morphine examined in parallel tests. Human monocytes

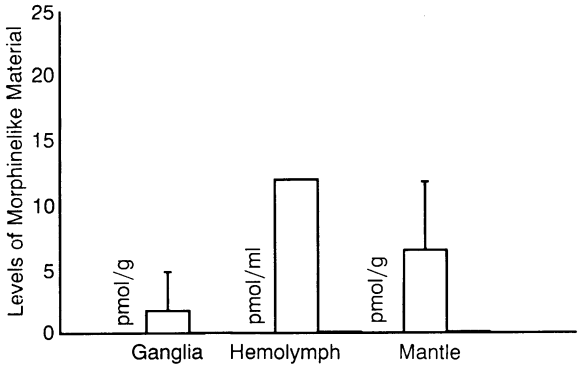

FIG. 2. Levels of morphine-like material in normal *Mytilus edulis* (mean ± SEM).

Table 1. Interference with TNF-α-induced chemotactic activity by morphine

Treatment	% cells exhibiting chemotaxis		
	Monocytes	*Mytilus*	*Leucophaea*
Control	39.0 ± 3.1	32.2 ± 0.5	23.1 ± 2.1
Morphine	3.1 ± 1.1	5.3 ± 2.4	4.1 ± 1.3
Morphine + naloxone	33.4 ± 5.2	27.4 ± 4.1	17.9 ± 3.3

Five viewing areas were observed on each slide, each noting the axis and shape of 46–57 cells per field. Data of each slide were combined with data of four other slides to provide the overall data of the five slides (mean ± SEM). Fields of view (400 μm^2) containing objects <1.5 μm in diameter or width were omitted because they were deemed to be bacteria and/or cellular debris. TNF-α (control), 10 units/ml; morphine, 1 μM; naloxone, 1 μM.

and *Mytilus* immunocytes incubated with increasing concentrations of morphine exhibited a dose-dependent decrease in their chemotactic ability (Table 1). This effect counteracted that of either TNF-α or IL-1α that had been added simultaneously to the incubation medium (10 units/ml). The same antagonistic influence of morphine on chemotactic activity was observed in a smaller number of immunoactive hemocytes of the insect *Leucophaea maderae* (Table 1).

The process of deactivation in the presence of morphine or morphine-like substances expressed itself in a lowering of cellular velocity in IL-1α-stimulated immune cells (Table 2). The HPLC fraction corresponding to morphine obtained from *Mytilus* ganglia showed the same effects in both human and invertebrate cell types (Table 2). Like that of authentic morphine (21), this chemotaxis-lowering effect was naloxone-sensitive (Table 2). Interestingly, codeine could mimic the effect of authentic morphine but only at much higher concentrations (10^{-5} to 10^{-4} M; data not shown). Thus, the RIA-positive codeine-like material obtained from the first isolation procedure, which allows for a more distinct separation of morphine-like and codeine-like substances, was without effect (Table 2).

Table 2. Inhibition of IL-1α-stimulated immunocyte velocity by morphine

Substance(s) tested	Velocity, μm/sec	
	Human monocytes	*Mytilus* immunocytes
Authentic material		
Control	0.07 ± 0.01	0.14 ± 0.07
IL-1	0.18 ± 0.03*	0.33 ± 0.03*
Morph + IL-1	0.06 ± 0.03	0.10 ± 0.08
Morph + IL-1 + Nal	0.15 ± 0.03*	0.30 ± 0.04*
Method 1		
Morph-like + IL-1	0.03 ± 0.02**	0.05 ± 0.03**
Morph-like + IL-1 + Nal	0.16 ± 0.04*	0.26 ± 0.06*
IL-1 + codeine-like	0.15 ± 0.04	0.30 ± 0.05
Method 2		
Morph-like + IL-1	0.04 ± 0.01**	0.06 ± 0.02**
Morph-like + IL-1 + Nal	0.15 ± 0.04*	0.28 ± 0/−0.05
Met-EK-like	0.16 ± 0.03	0.27 ± 0.05

Endogenous materials were obtained by methods 1 and 2. Concentrations of ligands used were as follows: IL-1α, 1 unit/ml; morphine sulfate, 10 nM; naloxone, 10 nM. Control values represent data from three experiments (20–31 cells per test; mean ± SEM). The 0- to 15-min determinations were omitted because both human and *Mytilus* immunocytes started with zero velocity. Mean velocity values represent readings taken at 15-min intervals for 1 hr. Morph, morphine; Nal, naloxone; Met-EK, Met-enkephalin. *, $P < 0.05$ compared to control; **, $P < 0.05$ compared to IL-1 stimulation of velocity.

Additional parameters examined were the degree of cellular adherence and changes in cellular conformation of *Mytilus* and *Leucophaea* immunocytes exposed to morphine. In immunocytes responding to the activating influence of TNF-α, the addition of morphine caused them to revert to a rounded conformation comparable to, but not identical with, that of the prestimulated state. The cellular area in TNF-α- or IL-1α-stimulated cells was 275 ± 15.1 μm^2 [form factor (ff), 0.61 ± 0.03], that of cells exposed to both TNF-α and morphine was 156 ± 9.8 μm^2 (ff, 0.87 ± 0.04), and that in nonstimulated cells was 101 ± 5.7 μm^2 (ff, 0.83 ± 0.03).

Opiate Receptors in *Mytilus* Immunocytes and Human Monocytes. The demonstration of the presence and pharmacological properties of morphine substances in *Mytilus* tissues was further substantiated by the identification of specific morphine receptor sites on this animal's immunocytes similar to those in human monocytes. Scatchard analysis revealed a single relatively high-affinity binding site with a K_d value of 42 nM and a B_{max} value of 2.03 pmol/g of membrane protein for monocytes and with a K_d value of 56.7 nM and a B_{max} of 2.33 pmol/g of membrane protein for *Mytilus* immunocytes (Fig. 3), by using 3DHM. In replicate assays with both cell types with glass-on-glass homogenization, the K_d value remained relatively constant, whereas the B_{max} value appeared to vary (2.03–7.88 pmol/g of protein).

A variety of opioids, tested by two methods, were found to be ineffective in displacing specifically bound 3DHM or [^{3}H]diprenorphine (Tables 3 and 4). In contrast, the opiate alkaloid μ ligands were relatively potent and κ ligands, dynorphin-(1–17) and ethylketocyclazocine, were quite weak. Naloxone was found to be less potent than naltrexone in counteracting this activity.

Role of Endogenous Opiates in Invertebrate Stress. To demonstrate possible functional roles of morphine-like material in *Mytilus*, we examined its presence in the course of a stressful situation brought about by experimental intervention (20). In animals subjected for 6 hr to electrical stimulation of the pedal ganglia and interference with valve movements, 77% ($P < 0.005$) of the hemocytes were in the ameboid-activated state (ff, 0.41 ± 0.08), as compared with only 5% in the unstressed controls. Thirty hours later the immunocytes showed a rounded conformation (ff, 0.78 ± 0.07), but their areas were larger than those of unstressed controls (167.3 ± 11.2 μm^2 versus 97.3 ± 9.4 μm^2 in the unstressed group; $P < 0.01$). These hemocytes resembled those of intact specimens having been exposed to morphine

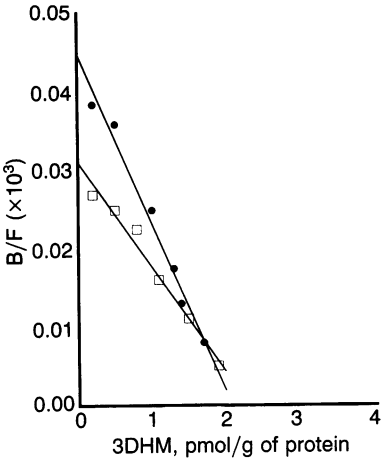

FIG. 3. Scatchard analysis of 3DHM binding to membrane suspensions of human monocytes (●) and *Mytilus* immunocytes (□) replicated three times. For human monocytes, K_d = 42 nM and B_{max} = 2.05 nM. For *Mytilus* immunocytes, K_d = 56.7 nM and B_{max} = 2.33 nM. B, bound; F, free.

Table 3. Displacement of 3DHM by opioid and nonopioid ligands in human monocyte and *Mytilus* immunocyte membrane suspensions

Ligand	Class	IC_{50}, nM: Human monocytes	IC_{50}, nM: *Mytilus* immunocytes
Agonist			
DAMA	δ	>1000	>1000
Deltorphin		>1000	>1000
Met-enkephalin		>1000	>1000
DADLE		>1000	>1000
DPDPE		>1000	>1000
DAGO	μ	>1000	>1000
DHM		45	62
Morphine		43	54
Etrophine		67	66
Dynorphin-(1–17)	κ	>1000	>1000
EKC		200	175
Antagonist			
Naloxone		420	545
Naltrexone		170	210

Aliquots of membrane suspensions from human monocytes and *Mytilus* immunocytes were incubated with nonradioactive compounds at six concentrations for 10 min at 22°C and then with 40 nM 3DHM for 60 min at 4°C. One hundred percent binding is defined as bound 3DHM in the presence of 10 μM dextrorphan minus bound 3DHM in the presence of 10 μM levorphanol. The IC_{50} value is defined as the concentration of drug that elicits half-maximal inhibition of specific binding. The mean ± SEM for three experiments is given. The displacement analysis data indicate the potency of various opioid extracts in displacing 3DHM and may give specific information on different receptor populations. Incubation medium for Met-enkephalin contained phosphoramidon (100 μM) and bestatin (100 μM) to inhibit enzyme action (16). DAMA, [D-Ala^2,Met^5]enkephalinamide; DADLE, [D-Ala^2,Leu^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin (where Pen is penicillamine); DAGO, [D-Ala^2,MePhe^4,Glyol^5]enkephalin; EKC, ethylketocyclazocine.

or to the HPLC fraction of the pedal ganglia containing morphine-like material (ff, 0.80 ± 0.08; area, 173.2 ± 9.3 μm^2).

Examination of the levels of endogenous morphine-like material revealed a significant increase in the hemolymph (56.8 ± 3.7 pmol/ml versus 12.1 ± 3.4 pmol/ml in the control) and in the pedal ganglia (167 ± 101 pmol/g versus 45 ± 10.2 pmol/g) taken from the 30-hr group. The 30-hr levels of codeine-like material (presumed precursor of morphine) after stress rose insignificantly to 0.65 pmol/g, as compared with the control value of 0.25 pmol/g in unstressed animals, suggesting that negative feedback does not occur in its presumed transformation to morphine.

Table 4. Displacement of [^{3}H]diprenorphine by opioid and nonopioid ligands

Ligand	Class	Dose, μM	Human monocyte-specific binding, fmol of [^{3}H]DPN per mg of protein
DADLE	δ	10	0.0 ± 2.3
DPN	μ	0.01	8.4 ± 2.8
DPN		10.0	25.0 ± 5.1
Morphine		0.01	7.8 ± 2.0
Morphine		10.0	23.8 ± 5.2
Cyclazocine	κ	10.0	5.4 ± 3.2

Cells were incubated with 2.1 nM [^{3}H]diprenorphine and centrifuged and the cell pellet was resuspended in 50 mM Tris·HCl (pH 7.5). The cells were disrupted and prepared as described elsewhere (22). DADLE, [D-Ala^2,Leu^5]enkephalin; DPN, diprenorphine.

DISCUSSION

The present report demonstrates that invertebrate tissues contain morphine-like and codeine-like substances considered to be endogenous. Information obtained in vertebrates indicates that the same or closely related opiates occur in several mammalian organs, including the brain (7–10, 14), and in amphibian skin (11). However, exact data on their functional activity and on their corresponding receptor sites are still missing.

Tests with the material extracted from the pedal ganglia of *Mytilus* revealed distinctive effects on human monocytes and invertebrate immunocytes resembling those of authentic morphine. More specifically, this activity is one of deactivation, antagonizing the simultaneous chemotaxis-stimulating activity of TNF-α and IL-1α, and expressing itself in characteristic conformational changes.

A single class of relatively high-affinity μ-like opiate binding sites was identified in both invertebrate and vertebrate immunocytes. This strongly suggests that this receptor site for opiate compounds may be used exclusively by immunocytes, since in invertebrate and mammalian neural tissues the reported μ sites do not exhibit such a high selectivity toward opiate substances (see ref. 6). Furthermore, the affinity constant of this binding site is close to the pharmacological dose of morphine that can significantly inhibit the cytokine-stimulated migratory activity, suggesting that in immune cells inhibition of chemotactic activity by morphine is mediated by this receptor. In summary, the present study has shown that the immune systems of mammals and *Mytilus* respond to opiate alkaloids in the same manner and by way of the same μ_3 opiate receptor.

The deactivating influence of morphine substances in both systems has to be interpreted in context with the body of information on immunoregulation, particularly, immunostimulating activities of opioid peptides and other messenger molecules. The question arises how these opiates, operating at significantly higher concentrations than, e.g., Met-enkephalin (16), participate in the interaction of the multiple signal molecules forming the immunoregulatory network. The existence and significance of a dual mechanism of control over immune-cell activity have been discussed in earlier studies (3). It has been reported (23) that, at concentrations considerably higher than those recorded for immunostimulation, certain neuropeptides may act as immunosuppressants. Enkephalinergic immunocyte activation can be downregulated by neutral endopeptidase 24.11 (NEP) (16, 24). Moreover, in response to immunocyte activation by either cytokines or neuropeptides, NEP is upregulated resulting in enhanced inhibition of cellular activity (16, 24). The concept that the immunosuppressive effect of endogenous morphine-like material is due to its capacity of upregulating NEP activity is supported by the observation that the amount of this enzyme, considered to decrease enkephalin activity, is selectively increased in mouse striatum chronically treated with morphine (25).

The need for a functional interaction of regulatory factors with opposite effects can be considered to be enhanced under abnormal conditions. The present data on experimentally induced stress in *Mytilus* indicate that, 30 hr after acute cellular response, the immunocytes become inactive concomitantly with higher opiate levels in the hemolymph and the ganglia. This suggests a general and yet specific role of morphine in calming or terminating the state of alertness created by the initial release of endogenous opioids and/or cytokines (20).

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