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Immunology

[D-Ala^2]Deltorphin I binding and pharmacological evidence for a special subtype of δ opioid receptor on human and invertebrate immune cells

(receptor binding/human granulocytes/*Mytilus edulis*/*Leucophaea maderae*)

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ABSTRACT **The effects of the opioid neuropeptide [D-Ala^2]deltorphin I, isolated from amphibian skin, on immunoregulatory activities were studied in representatives of vertebrates and invertebrates. The high potency of this compound parallels that of [Met]enkephalin, which was previously demonstrated in vertebrate plasma and invertebrate hemolymph. The addition of [D-Ala^2]deltorphin I at 10^{-11} M to human granulocytes or immunocytes of the mollusc *Mytilus edulis* resulted in cellular adherence and conformational changes indicative of cellular activation. This value is in line with the concentrations obtained with [Met]enkephalin, tested in the presence of the specific neutral endopeptidase 24.11 inhibitor phosphoramidon, and this opioid's synthetic analog [D-Ala^2, Met^5]enkephalin which, like [D-Ala^2]deltorphin I, is resistant to proteolytic degradation. Both ligands appear to be acting on the same population of immunocytes. The same relationship was estimated to exist in the insect *Leucophaea maderae*, in which the high viscosity of the hemolymph makes the quantification of reactive cells more difficult than in *Mytilus*. In addition, [D-Ala^2]deltorphin I is as potent as β-endorphin in affecting the proliferation of lymphocytes in response to mitogen. Saturation experiments with unlabeled ligands and the radioligands [^{3}H][D-Ala^2]deltorphin I and [^{3}H][D-Ala^2,Met^5]enkephalinamide revealed the presence of two high-affinity binding sites on human granulocytes, one sensitive to the nonequilibrium δ opioid antagonist [D-Ala^2,Leu^5,Cys^6]enkephalinamide and the other relatively insensitive. The results obtained with [D-Ala^2]-deltorphin I support the view that the special role played by endogenous [Met]enkephalin in immunobiological activities of vertebrates and invertebrates is mediated by a special subtype of δ opioid receptor.**

The inflammatory responses generated by immunoactive cells in vertebrates and invertebrates have certain features in common. Previous studies have shown that the activity of human granulocytes and their counterparts in higher invertebrates is modulated by neuropeptides, especially opioids (see refs. 1 and 2). Evidence for the existence of a dose-dependent stimulatory effect of several of these molecules has been obtained by the observation of conformational changes in these immunocytes (flattening, development of pseudopodia) and their movement in a directed manner (3–7). Moreover, these opioid-mediated effects were found to be inhibited by naloxone, indicating that they result from the specific interaction with opioid receptors (5, 6). The high potency of one of the opioids tested, [Met]enkephalin, which is known to be present in vertebrate plasma and invertebrate hemolymph, suggested the existence of a novel subtype of δ receptor for this and possibly other opioid ligands in the immune system (6).

The present study deals with a series of comparable tests carried out with the endogenous amphibian heptapeptide [D-Ala^2]deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2). It was undertaken because deltorphins, isolated from skin extracts of the frog *Phyllomedusa bicolor*, have been shown to have high affinity and selectivity for δ opioid binding sites (8). The data for [Met]enkephalin used for comparison with those for [D-Ala^2]deltorphin I had to be obtained as follows: One set of tests was carried out with the synthetic analog of [Met]enkephalin [D-Ala^2,Met^5]enkephalinamide (DAMA), which is resistant to the down-regulating effect of the naturally occurring neutral endopeptidase 24.11 (NEP) (7, 9). The other test, with [Met]enkephalin, required the addition of the specific enzyme inhibitor phosphoramidon to yield the same high potency values as those with DAMA (7). The results of the corresponding tests carried out with [D-Ala^2]deltorphin I were the same in the presence or absence of an enzyme inhibitor. They clearly indicate that [D-Ala^2]deltorphin I is at least as effective as [Met]enkephalin in stimulating the activity of immunocytes as well as the proliferation of lymphocytes (10). Thus the present study provides additional evidence for the existence of a special δ opioid receptor in the immune system of vertebrates as well as invertebrates.

MATERIALS AND METHODS

Human granulocytes for cellular analysis were obtained from Long Island Blood Services (Melville, NY) and prepared as previously noted in detail (6, 7, 9). Invertebrate hemolymph was collected from the mussel *Mytilus edulis*, as previously described (5, 6). In addition, hemocytes from the insect *Leucophaea maderae* were obtained by allowing a drop of hemolymph, collected by an incision in the abdomen, to fall on an albumin-coated slide. Hemolymph and hemocytes were then rapidly mixed, by means of a small glass rod, with various osmotically balanced drugs or with vehicle alone, and a coverslip was placed on the preparation (5, 6).

Analysis of Cellular Activity. The mixture of human or invertebrate cells and ligands was allowed to incubate (23°C for invertebrates and 37°C for human cells) for 20 and 10 min, respectively, and prepared for light microscopic inspection and Zeiss-Zonax reflectance measurement (5). The numbers of cells per ×100 field were quantified as previously noted (5). Reflectance is a relative measure of light reflecting off the

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Abbreviations: DAMA, [D-Ala^2,Met^5]enkephalinamide; DALCE-NH_2, [D-Ala^2,Leu^5,Cys^6]enkephalinamide; DPDPE, [D-penicillamine^{2,5}]enkephalin; NEP, neutral endopeptidase 24.11.

surface of the cells during epi-illumination. The higher the value for light measured, the greater the reflectance and the greater the number of cells in the field of analysis (0.4 mm^2). Cellular debris does not interfere with the results, since a large number of readings per slide are taken. The value obtained is the mean from 10–20 readings per slide replicated four times and analyzed by the Student *t* test. The reflectance value is relative to the degree of cell clumping in invertebrates. This value is adjusted, because it is relative to the clumping of control cells, by controlling the size of the "pin hole" before light hits the Hammatsu photomultiplier.

Reagents, including [D-Ala^2]deltorphin I, were added to the incubation medium, either alone or together with the opiate antagonist naloxone. Following incubation, preparations were examined microscopically as described elsewhere in detail (5, 6). Changes in cellular conformation, which ranged from inactive–rounded to active–ameboid, were determined by measurements of cellular area and perimeter and were mathematically expressed by use of the shape-factor formula of the American Innovision (San Diego) analysis system, $A_C/A_T = (L_T/L_C)^2$, where A_T is the area of a circle with the same perimeter as that of a given cell and L_T is the perimeter of a circle with the same area as that of a given cell, and A_C and L_C represent the actual area and perimeter of the cell (11). The lower this number, the higher the cellular perimeter and the more ameboid the cellular shape. The proportion of activated cells was determined as noted elsewhere in detail (5). Activated cells not only change their conformation in response to a pharmacological stimulus (e.g., [Met]enkephalin), they also become mobile and are capable of phagocytosis.

Binding Experiments. Human granulocytes for binding analysis were obtained from volunteer donors at the AVIS Blood Service (Rome) and Long Island Blood Service, and processed as previously described by Erspamer *et al.* (8).

[^{3}H][D-Ala^2]deltorphin I binding was performed in 50 mM Tris·HCl, pH 7.4, at 35°C without enzyme inhibitors for 90 min. Each point represents the mean of 12 experiments made with four different membrane preparations. In these "cold saturation" experiments a set concentration (0.2 nM) of radioligand is added to each tube and the concentration of the ligand is increased by adding progressively larger amounts of unlabeled ligand. *Mytilus* binding assays were performed as previously noted in greater detail (12).

Covalent binding of [D-Ala^2,Leu^5,Cys^6]enkephalinamide (DALCE-NH_2) (13) to human granulocyte opioid receptors was performed by incubating membranes for 60 min at 35°C in 50 mM Tris·HCl, pH 7.4, containing DALCE-NH_2 (2–20 μM), 100 mM NaCl, 5 mM $MgCl_2$, 2 μM GTP, 100 μM phosphoramidon, 100 μM captopril, bacitracin at 100 μg/ml, and bestatin at 10 μg/ml. Control membranes preincubated in the absence of DALCE-NH_2 received an aliquot of the acid solvent used to dissolve DALCE-NH_2 equal to that added to DALCE-NH_2-treated membranes. Following preincubation, membranes were centrifuged at 37,000 × *g* for 10 min, resuspended in an equal volume of ice-cold 50 mM Tris·HCl, pH 7.4, and centrifuged again. The pellet was resuspended in an equal volume of dissociation buffer and incubated for 60 min at 35°C. Dissociation buffer consisted of 50 mM Tris·HCl at pH 7.4, 250 mM NaCl, 100 μM guanosine 5′-[β,γ-imido]triphosphate, and the protease inhibitors described above. The membranes were then washed twice with ice-cold Tris buffer, resuspended to half the original volume in 50 mM Tris·HCl, pH 7.4, and stored at −70°C for binding assays. To prevent dimerization, peptide was stored at −25°C in 0.12 M HCl containing 20 mM 2-mercaptoethanol (acid solvent). DALCE-NH_2 was synthesized by the solid-phase technique with fluoren-9-ylmethoxycarbonyl (Fmoc) strategy. [^{3}H][D-Pen^{2,5}]Enkephalin, ([^{3}H]DPDPE), in which D-Pen represents D-penicillamine, was from Bachem. Other ligands listed in Table 1 were from Peninsula Laboratories. Mathematical analysis of the data and details of the binding assay were as described by Erspamer *et al.* (8).

For IC_{50} determination (defined as the concentration of drug that elicits half-maximal inhibition of specific [^{3}H]DAMA binding), aliquots of human granulocyte-membrane suspension (prepared from cells obtained from Long Island Blood Services) were incubated with nonradioactive opioid compounds at six different concentrations for 10 min at 22°C and then with [^{3}H]DAMA for 60 min at 4°C as previously noted in detail (12). The mean ± SEM for three experiments is recorded for each compound tested.

Proliferation Assay. Mouse spleen cells (C3H/HeJ), 1×10^6 per well, were treated with concanavalin A (Con A; 2.5 μg/ml) and the indicated concentrations of [D-Ala^2]deltorphin I. After 2 or 4 days of incubation, 1 μCi (37 kBq) of [^{3}H]thymidine was added to the culture. Twenty-four hours later, the cells were harvested, DNA was precipitated onto glass filters, and radioactivity incorporated into the DNA measured by standard liquid scintillation counting techniques (10). Values reflect the average of duplicate cultures ± SD.

RESULTS

[D-Ala^2]Deltorphin I has the ability to stimulate, in a naloxone-sensitive manner, both human and invertebrate immunocytes (Fig. 1*A*). Concomitant presence of DAMA did not increase the number of cells responding to [D-Ala^2]deltorphin I alone, indicating that both ligands appear to be acting on the same population of immunocytes and presumably on the same type of receptor.

A smaller number of immunocytes of the insect *Leucophaea* than those of *Mytilus* or humans appear to be responsive to either ligand (Fig. 1*A*). However, it must be mentioned that the hemolymph of *Leucophaea* is extremely viscous and tends to coagulate upon exposure to air. This condition seems to hinder conformational changes of cells (elongation) as well as their mobility (5) and makes the quantification of the cellular response difficult.

Other physical parameters of human and *Mytilus* immunocyte responsiveness to [D-Ala^2]deltorphin I (immunocyte adherence, area increase, and shape factor) all demonstrate that [D-Ala^2]deltorphin I is very potent, exerting peak effects at 10^{-11} M (Fig. 1 *B*, *C*, and *D*). Indeed, the compound is equipotent to DAMA as well as [Met]enkephalin, the latter being tested in the presence of the NEP inhibitor phosphoramidon (7). It was of interest to determine if certain specific peptide inhibitors would potentiate the pharmacological effects of [D-Ala^2]deltorphin I on immunocyte responsiveness. During the time course of our experiments, phosphoramidon (NEP inhibitor, 100 μM) and captopril (aminopeptidase inhibitor, 100 μM) were found to be ineffective (Fig. 2). This result supports the conclusion reached by Erspamer *et al.* (8) that this compound is not readily degraded by enzymes.

In addition, it was important to demonstrate that [D-Ala^2]-deltorphin I was just as potent in a more conventional assay as it was in our assay for conformational responses of immunocytes. Thus, we determined whether [D-Ala^2]deltorphin I would affect proliferation of lymphocytes in response to mitogen as previously demonstrated for β-endorphin (14). Mouse spleen cells were treated with the T-cell mitogen Con A, in the absence or presence of [D-Ala^2]deltorphin I at concentrations ranging from 10^{-5} to 10^{-11} M, and assayed for [^{3}H]thymidine incorporation at 2 and 4 days. As shown in Fig. 3, [D-Ala^2]deltorphin I at all concentrations tested enhanced Con A-induced spleen-cell proliferation. The effect was roughly biphasic and was more pronounced at lower concentrations. The effect of [D-Ala^2]deltorphin I, especially at the lower concentrations (10^{-9} to 10^{-11} M), appeared to be lasting, since the enhancement was still evident after 4 days (Fig. 3). This result indicates that [D-Ala^2]deltorphin I not only is po-

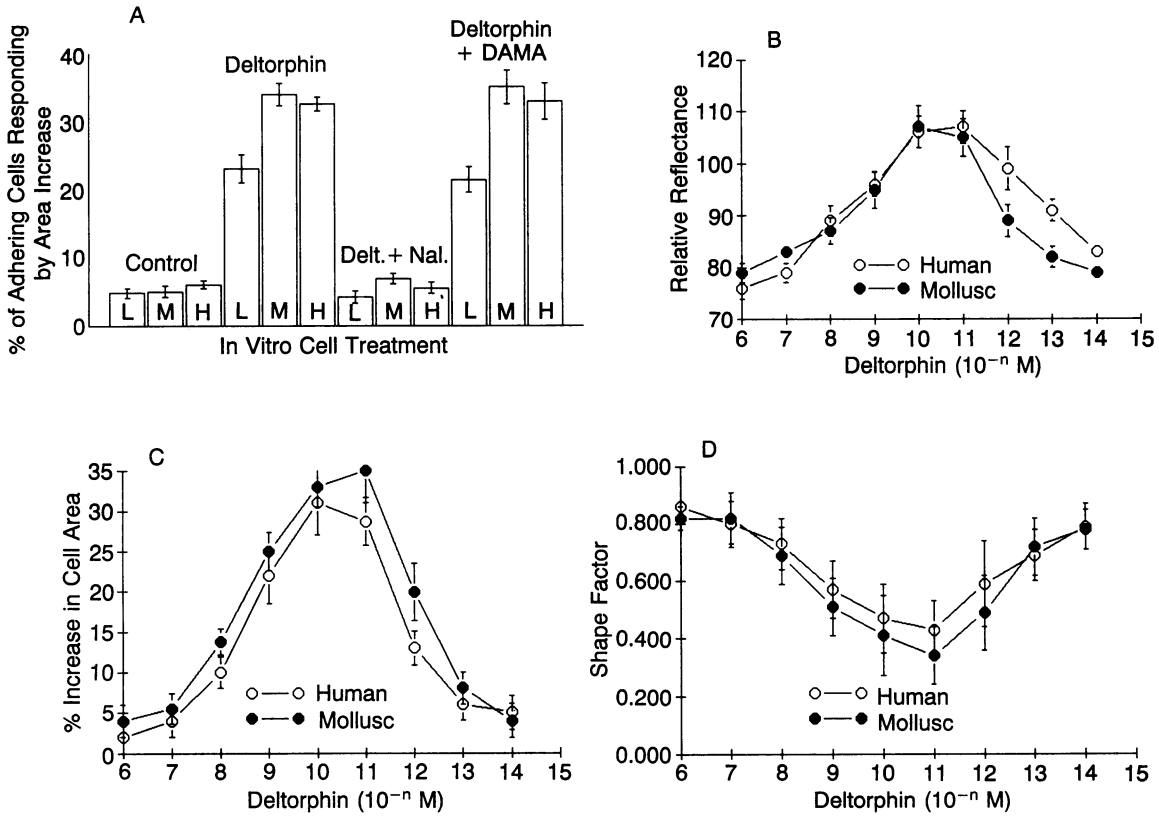

FIG. 1. Comparison of stimulatory effects of [D-Ala²]deltorphin I (10^{-11} M) on *L. maderae* and *M. edulis* immunocytes and human granulocytes. (*A*) Increase in cellular size, indicative of activation in both humans (H) and invertebrates (L, M) in a naloxone (Nal.)-sensitive manner and in combination with DAMA. (*B*) Increased adherence of human granulocytes and *Mytilus* immunocytes to albumin-coated slides, measured by relative reflectance. (*C* and *D*) Conformational changes of activated human granulocytes and *Mytilus* immunocytes illustrated by cellular area (*C*) and shape-factor analysis (*D*). Each bar or point represents the mean of 20–50 readings ± SD.

tent but also has a persistent activity in this proliferation assay.

Given the efficacy of [D-Ala²]deltorphin I in immune systems of diverse animals, we sought to biochemically characterize the opioid receptor presumably involved in this activity. In "cold saturation" experiments, where a set concentration (0.2 nM) of radioligand ([³H][D-Ala²]-deltorphin I) is added to each tube and the concentration of the ligand is increased by adding progressively larger amounts of unlabeled ligand, a single high-affinity binding site was revealed with a K_d of 0.45 nM and a B_{max} of 3.78 pmol/g of membrane protein for human granulocytes (Fig. 4*A*). Hill analysis revealed a coefficient of 0.967 (Fig. 4*B*).

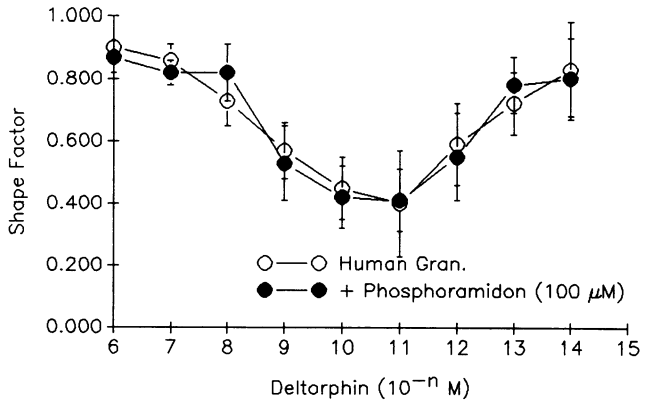

FIG. 2. Lack of potentiation of [D-Ala²]deltorphin I potency by the specific NEP inhibitor phosphoramidon. Measurements were performed as indicated for Fig. 1*D*.

In *Mytilus* immunocyte membrane suspensions a single high-affinity [³H]DAMA binding profile became evident upon Scatchard analysis (Fig. 5*A*). This high-affinity site has a K_d of 0.8 nM and a B_{max} of 4.0 pmol/g of protein. Interestingly, in repetitive assays with glass-on-glass homogenization the K_d remained relatively constant, whereas the B_{max} appeared to vary (0.9–8.0 pmol/g; data not shown). The same is true of the human granulocyte B_{max} (2.0–8.0 pmol/g). Furthermore, when a Brinkmann Polytron was used to homogenize the various cells we frequently were unable to find specific binding. This demonstrates that immunocyte opioid recep-

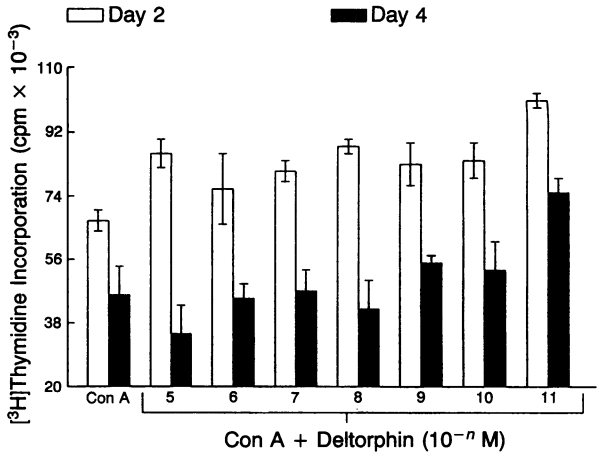

FIG. 3. Effects of [D-Ala²]deltorphin I on Con A-induced mitogenesis in C3H/HeJ mouse splenocytes as measured by [³H]thymidine incorporation on day 2 and day 4.

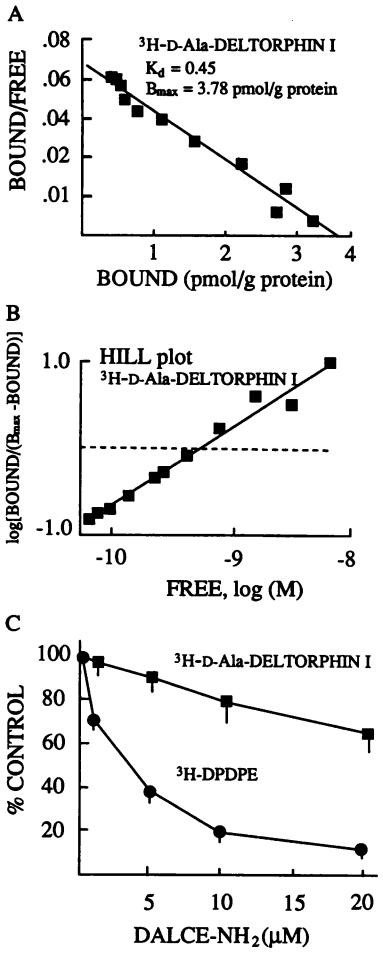

FIG. 4. Binding of [^{3}H][D-Ala^2]deltorphin I to human granulocytes. (*A*) Scatchard analysis. K_d and the concentration of free ligand are in nM. (*B*) Hill analysis. (*C*) Displacement analysis.

tors may be extremely sensitive to physical disturbances. It may also explain why certain investigators have been unable to demonstrate highly specific stereospecific opioid binding sites (see ref. 1).

Figs. 4*C* and 5*B* show the effects of preincubation of human granulocyte membranes with increasing concentrations of DALCE-NH$_2$, a nonequilibrium δ opioid antagonist, on recovery of the δ binding sites of [^{3}H]DPDPE, [^{3}H][D-Ala^2]deltorphin I, and [^{3}H]DAMA in *Mytilus* granulocytes. DALCE-NH$_2$ pretreatment resulted in a marked concentration-dependent decrease in recovery of [^{3}H]DPDPE-binding sites in both cell types, whereas it only slightly modified the recovery of [^{3}H][D-Ala^2]deltorphin I-binding sites in human granulocytes. However, in *Mytilus* immunocytes a smaller decrease in displaceable sites was observed (Fig. 5*B*).

The ability of a variety of other opioids to displace specifically bound [^{3}H]DAMA was investigated in another experiment (Table 1). The opioid peptides were effective in the following decreasing order: [D-Ala^2]deltorphin I = DAMA > [Met]enkephalin > DADLE > DPDPE. By contrast, the μ and κ ligands DAGO and dynorphin-(1–17) were quite weak. Naltrexone was found to be more potent than naloxone in displacing [^{3}H]DAMA.

DISCUSSION

In comparing the immunomodulatory effects of the two opioid peptides under consideration here, two facts have to be taken into account. The occurrence of [D-Ala^2]deltorphin

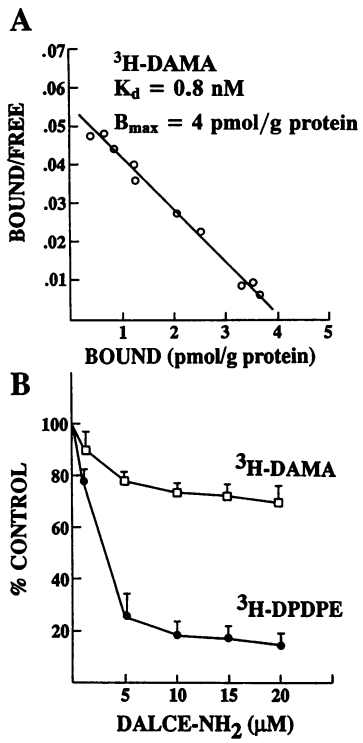

FIG. 5. Binding of [^{3}H]DAMA to *Mytilus* immunocyte membrane suspensions. (*A*) Scatchard analysis. (*B*) Displacement analysis.

I or a deltorphinlike substance in the immune system has not been demonstrated, and this peptide, like the exogenous [Met]enkephalin analog DAMA, is resistent to proteolytic attack by NEP and aminopeptidase (16). Therefore, the high potency values for [D-Ala^2]deltorphin I, observed in tests with immunocytes of vertebrates and invertebrates, are in line with those for DAMA and those for [Met]enkephalin in the presence of the NEP inhibitor phosphoramidon (7). Furthermore, our results in the Scatchard analysis indicate that the binding sites for [D-Ala^2]deltorphin I on human granulocytes, like those for DAMA on both human and *Mytilus* granulocytes, are of a single type and of high efficiency. However, a high-affinity site appears to exist that is

Table 1. Displacement of [^{3}H]DAMA (1 nM) by opioid ligands in human and *Mytilus* immunocyte membrane suspensions

Ligand	IC_{50}, nM	
	Human granulocytes	*Mytilus* immunocytes
δ		
DAMA	0.6	0.6
[D-Ala^2]Deltorphin I	0.5	0.5
[Met]Enkephalin*	0.7	0.9
DADLE	6.2	7.7
DPDPE	8.1	10.4
μ		
DAGO	12.3	16.5
κ		
Dynorphin-(1–17)	21.2	19.3
Antagonists		
Naloxone	15.6	22.7
Naltrexone	1.1	1.0

DADLE, [D-Ala^2,Leu^5]enkephalin; DAGO, [D-Ala^2,MePhe^4, Gly(ol)^5]enkephalin.

*Incubations with [Met]enkephalin contained phosphoramidon (100 mM) and bestatin (100 μM) to inhibit enzyme action (7, 9, 15).

relatively insensitive to naloxone, as noted in the displacement assays for both cell types.

Thus, the present results obtained with [D-Ala^2]deltorphin I provide additional support for the concept, based on earlier studies (6), that a special δ subtype of opioid receptor for immunoregulating [Met]enkephalin exists on human granulocytes and their counterparts in invertebrates. It has been previously demonstrated that pretreatment of rat brain membranes with DALCE-NH_2 leads to loss of DPDPE-binding sites in a concentration-dependent manner (13). In the present studies, pretreatment of granulocyte membranes with increasing concentrations of DALCE-NH_2 significantly blocked [^{3}H]DPDPE binding to δ opioid sites. By contrast, the same treatment of granulocyte membranes with DALCE-NH_2 did not affect the binding of [^{3}H][D-Ala^2]deltorphin I and [^{3}H]DAMA. We, therefore, propose that the special opioid receptor postulated to interact with [Met]enkephalin in its immunomodulatory function be tentatively classified as a subtype, δ_2, of the classical δ receptor, δ_1.

In examining the modulatory role played by opioid neuropeptides in another biological activity—i.e., growth—Zagon *et al.* (15, 17, 18) likewise found [Met]enkephalin to be the most potent among those tested. The special growth-related opioid receptor involved (referred to by these investigators as ζ receptor) was found to be abundant in the cerebellum of infants and in a human brain tumor, but not in the cerebellum of normal adults. Using a tissue culture of murine S20Y neuroblastoma, they demonstrated that [Met]-enkephalin exhibited the most potent action in inhibiting growth. [Met]Enkephalin was more potent than [Met]enkephalin-Arg-Gly-Leu and [Met]enkephalin-Arg-Phe, as well as the synthetic δ ligands DPDPE and ICI 174,864. μ, κ, and ε ligands were without effect.

As to the functional significance of this putative subtype of δ receptor, we propose a dynamic association with NEP (CD10, enkephalinase), the specific enzyme modulating the stimulatory effect of [Met]enkephalin on the inflammatory response (7, 9). This view is supported by the extremely low binding site density for [D-Ala^2]deltorphin I and DAMA in these immunocytes. The low density of this receptor, and the high fidelity of signal recognition regulated by NEP, may be considered to be advantages in accommodating additional signal molecules controlling these immunoactive cells. In other words, this special δ receptor may be instrumental in the "stereospecific fine tuning" of immunoregulatory signals.

The close hypothetical association of immunocyte neuropeptide receptors with NEP can be noted in a recent report by Shipp *et al.* (9). Tumor necrosis factor stimulation of human granulocyte-bound NEP resulted in the immediate up-regulation of NEP activity. This enhancement of NEP activity caused the granulocytes to down-regulate their responsiveness to subsequent neuropeptide NEP substrates acting as signal molecules (19). This latter down-regulated condition did not totally inhibit further stimulation by NEP peptide substrates; it just made it more difficult to occur. Clearly, this appears to be a very effective method of modulating signal strength on the basis of concentration. It also allows the cell to establish a priority of response based on the first signal molecule that activates it, with a secondary mechanism to override the first if the stimulus is strong enough. These characteristics of granulocyte recognition and subsequent activation are critical for this cell, since many different peptidergic signals have the potential to have an impact on each single cell (15). It is of interest that this system appears to have been conserved in the course of a long evolutionary history (18, 20).

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