Biochemical Pharmacology, Vol. 43, No. 3, pp. 503–506, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

RECIPROCAL EFFECTS BETWEEN OPIOID PEPTIDES AND HUMAN POLYMORPHONUCLEAR LEUKOCYTES—II

ENHANCEMENT OF PHORBOL MYRISTATE ACETATE-INDUCED RESPIRATORY BURST IN HUMAN POLYMORPHONUCLEAR LEUKOCYTE BY OPIOID PEPTIDES PREVIOUSLY EXPOSED TO ACTIVATED OXYGEN SPECIES

AMAL SLAOUI-HASNAOUI, MARIE-CHRISTINE GUERIN, CHRISTIAN LE DOUCEN, JACQUES LOUBATIERE and JEAN TORREILLES*

INSERM U 58, 60 Rue de Navacelles, 34090 Montpellier, France

(*Received* 8 *July* 1991; *accepted* 8 *October* 1991)

Abstract—Activated oxygen species (AOS) have often been shown to promote strong modifications in peptide structures and thus in their biological functions. In the present study, the immunomodulatory effects of Leu-enkephalin, β-endorphin, dynorphin and some fragments are evaluated, before and after exposure of peptides to AOS, by studying their influence on human polymorphonuclear leukocyte (PMN) respiratory burst. None of the tested opioid peptides (modified or not) were shown to affect resting oxidative metabolism in the PMNs. The effects of peptides on phorbol myristate acetate (PMA)-stimulated production of AOS were measured in a lucigenin-enhanced chemiluminescence assay. Before AOS exposure, the opioid peptides suppressed the PMA-stimulated respiratory burst in human PMNs and a U-shaped dose–response relationship was observed. Conversely, after AOS exposure the opioid peptides enhanced the PMA-stimulated respiratory burst in human PMNs and an inverted U-shaped dose–response relationship was observed. In both cases, the maximal effect was reached at peptide concentrations of 10^{-10} M–10^{-12} M.

The enkephalins form a group of opioid peptides derived by processing a series of precursors including pro-opiomelanocortin, prodynorphin and proenkephalin [1]. Such precursor peptides are apparently synthesized in a number of tissues, such as the adrenal medulla, brain and intestinal mucosa, resulting either in the Leu-enkephalin (L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-leucine†) or Met-enkephalin pentapeptides or, alternatively, in intermediate fragments.

These peptides have been reported to function not only as analgesics in the central nervous system but also in the immunoregulatory processes in plasma, where they bind to specific receptors on human phagocytic leukocytes [2–4]: Diamant *et al.* [5] have shown that β- and γ-endorphins and their non-opioid derivatives suppress PMA-induced respiratory burst in human PMNs. β-Endorphins induce monocyte chemotaxis [6, 7], depress lymphocyte proliferation and enhance natural killer cell function [8].

Moreover, recent findings that leukocytes produce and secrete many peptide hormones such as endorphins, enkephalins, adrenocorticotropic hormone and arginine-vasopressin provide further evidence of the involvement of morphinomimetic peptides in immunomodulation (Ref. 9 and references within).

Since neutrophils are found virtually everywhere in human tissues, especially at inflammatory sites where they readily degranulate to release oxidative agents [10, 11], and enkephalin precursor peptides have been found to circulate in plasma that is apparently destined for these same sites [12], we attempted to determine whether respiratory burst in phagocytes might be modulated by opioid peptides.

We have shown, in the preceeding paper [13], that opioid peptides are hydroxylated by hydroxyl radicals generated either by PMA-stimulated PMNs, or by metal-catalysed ascorbate autoxidation leading to the formation of hydroxylated Leu-enkephalin with a tyrosyl-residue, instead of a phenylalanyl-residue at position 4 of the N-terminal part.

Thus, to determine whether hydroxylated opioid peptides affect the respiratory burst of PMA-stimulated PMNs, we compared their CL (using lucigenin as luminogen) in the presence of Leu-enkephalin and of a synthetic peptide YGGYL used as a model of hydroxylated opioid peptide.

MATERIALS AND METHODS

Reagents. β-Endorphin, Leu-enkephalin, dynorphin, FGGFL, YGGF, FGGF and GGFL were obtained from Bachem Feinchemikalein AG (Bubendorf, Switzerland) and tested for purity by reverse

* Corresponding author. Tel. (33) 67-54-15-44; FAX, (33) 67-52-06-77.

† Abbreviations: AOS, activated oxygen species; PMN, polymorphonuclear leukocyte; PMA, phorbol myristate acetate; CL, chemiluminescence; Leu-enkephalin, L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-leucine; Met-enkephalin, L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-methionine; YGGF, L-tyrosyl-glycyl-glycyl-L-phenylalanine; FGGF, L-phenylalanyl-glycyl-glycyl-L-phenylalanine; GGFL, glycyl-glycyl-L-phenylalanyl-L-leucine; YGGYL, L-tyrosyl-glycyl-glycyl-L-tyrosyl-L-leucine; FGGFL, L-phenylalanyl - glycyl - glycyl - L - phenylalanyl - L - leucine; GGYL, glycyl-glycyl-L-tyrosyl-L-leucine.

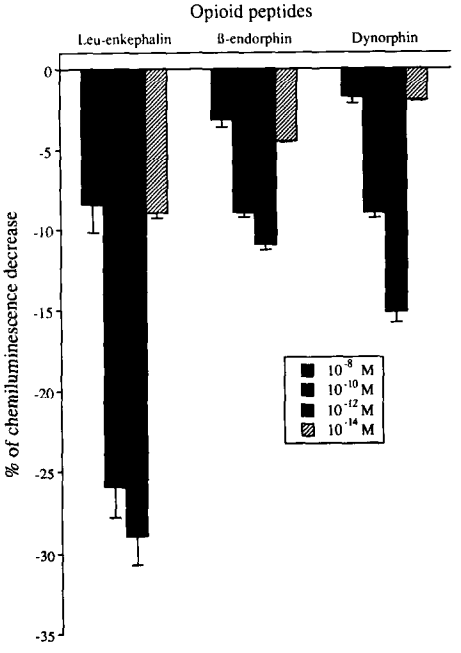

Fig. 1. CL inhibition of PMA-stimulated PMNs by opioid peptides. The effect of cell incubation with opioid peptides on the respiratory burst of PMNs is expressed as a percentage of the total CL response (area under the curve) elicited by PMA on 10^6 cells in the absence of peptide incubation. FGGFL and GGFL are without effects. The means and SD of 14 separate experiments are shown.

phase HPLC. YGGYL was prepared by Altergen (Strasbourg, France). PMA was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); stock solutions at a concentration of 1 mg/mL dimethyl sulfoxide were stored at −20° and diluted to 0.1 μg/mL before use. Modification of opioid peptides was conducted by reaction at room temperature in phosphate buffer (10 mM) pH 5 with a mixture containing 3 mM ascorbate, 5 mM peptide and 2 mM cupric sulfate in a total volume of 1 mL as described in the preceeding paper [13]. Cells were prepared from human peripheral blood obtained from healthy volunteer donors as described previously [13].

Lucigenin-enhanced CL assays. CL was monitored in a model 1251 luminometer LKB Wallac (Wallac Co, Turku, Finland) connected to an Apple computer in the presence of 0.5 mM lucigenin. PMA (0.1 μg/mL) was used as a stimulus. PMNs (10^6 cells) were incubated with peptides for 7 min, unless otherwise stated, at 37° before the addition of PMA. Measurements were carried out once a minute until a maximal CL value was obtained. Controls were performed with cells incubated without stimuli.

RESULTS

PMNs incubated with Leu-enkephalin and related peptides

Figure 1 summarizes the average values of the CL produced by addition of PMA to human PMNs, incubated previously with Leu-enkephalin and related peptides. (Because of an inter-donor variation in absolute peak CL values observed in PMNs of the different donors tested, the CL responses obtained in the presence of peptides were expressed as percentages of the CL response elicited by PMA stimulation of PMNs incubated under the same conditions as described in Materials and Methods, but without any peptides, and measured by the area under the curve. The deviation from these mean values never exceeded 12%.)

Leu-enkephalin and related peptides dose-dependently inhibited PMA-induced respiratory burst in human PMNs at concentrations ranging from 10^{-10} to 10^{-14} M without significant modification in the kinetics of the CL response; weaker effects correspond to the higher and lower concentrations used, giving rise to a U-shaped dose–response relationship.

In all cases, the obtained patterns were very similar to those of PMA-stimulated PMNs incubated without peptides. In the absence of the soluble PMA stimulus, opioid peptides did not significantly affect the basal CL response of PMNs.

PMNs incubated with YGGYL

As with the non-hydroxylated peptides, there was no significant change in kinetics of the CL response in PMN stimulated with PMA after incubation with YGGYL. But a dose-dependent enhancement of the PMA-induced respiratory burst was observed at concentrations of 10^{-10} to 10^{-14} M and an inverted U-shaped dose–response relationship was obtained (Fig. 2).

PMNs incubated with opioid peptides previously treated with ascorbate/Cu (II)

Since we have shown in the preceeding paper that the ascorbate/Cu (II) system hydroxylated opioid peptides, we incubated, for comparison, PMNs with the reaction mixture of some opioid peptides with AOS generated by the ascorbate/Cu (II) system. In all cases, we obtained a dose-dependent enhancement of the CL at concentrations of 10^{-10} to 10^{-14} M as observed with the model peptide YGGYL. (Controls performed under the same conditions with ascorbate or Cu (II) showed no significant effects on the CL response of the cells.)

PMNs incubated with non-opioid peptides previously treated with ascorbate/Cu (II)

When PMNs were incubated, before stimulation, with FGGFL treated with ascorbate/Cu (II) or with the synthetic peptide a similar enhancement of the CL response was observed (Fig. 2), whereas GGFL treated with the ascorbate/Cu (II) system remained without effect on the PMN CL; thus, tyrosine N-terminal residue seemed to be essential for the binding of peptides. This assumption was tested by the time–response study reported in Table 1 where CL of PMA-stimulated PMNs was measured as a function of the incubation duration with Leu-enkephalin treated with the ascorbate/Cu (II) system. A maximum of 15 min was observed, corresponding to the incubation period necessary for enzymatic cleavage of the N-terminal tyrosine residue by cell membrane-bound aminopeptidase at 37° [14].

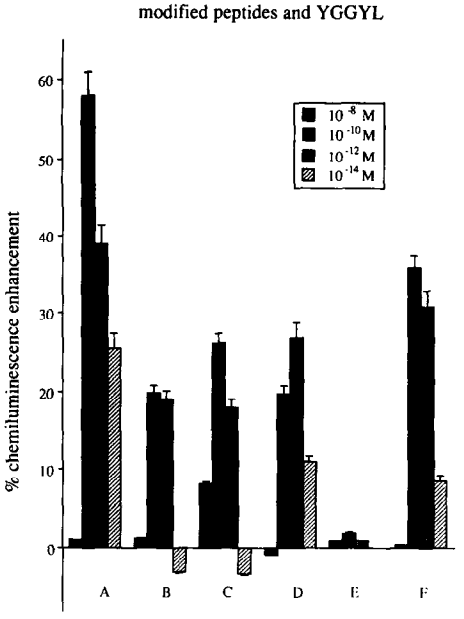

Fig. 2. CL enhancement of PMA-stimulated PMNs by opioid peptides and derivatives modified by exposure to AOS and by the model synthetic peptide YGGYL. The effect of cell incubation with the different peptides tested on the respiratory burst of PMNs is expressed as a percentage of the total CL response (area under the curve) elicited by PMA on 10^6 cells in the absence of peptide incubation. (A) Modified Leu-enkephalin; (B) modified dynorphin; (C) modified endorphin; (D) modified YGGF; (E) modified GGFL; (F) modified FGGFL or YGGYL. The means and SD of 14 separate experiments are shown.

Table 1. CL enhancement of PMA-stimulated PMNs (10^6 cells) by Leu-enkephalin (10^{-10} M and 10^{-12} M) after exposure to AOS

Incubation time (min)	Peak CL values (% of control) 10^{-12} M	10^{-10} M
1	50	36
3	58	39
5	54	31
10	46	23
12	31	17
15	23	7
20	8	2

CL values are a function of the incubation time of cells with peptides, before PMA stimulation. Controls were performed with cells incubated without peptides.

Study of the saturation of the binding sites of hydroxylated and non-hydroxylated peptides

Figure 3 shows the effects of incubation with mixtures of different concentrations of Leu-enkephalin and YGGYL on the lucigenine-enhanced CL of PMA-stimulated PMNs. When Leu-enkephalin 10^{-10} M was mixed with YGGYL 10^{-8} M the inhibition of the CL observed was the same as the inhibition obtained with Leu-enkephalin 10^{-10} M alone. Similarly, the enhancement of the CL observed when YGGYL 10^{-10} M was mixed with Leu-enkephalin 10^{-8} M was identical to the enhancement obtained with YGGYL 10^{-10} M alone showing that the binding sites of hydroxylated and non-hydroxylated peptides could be independently saturated.

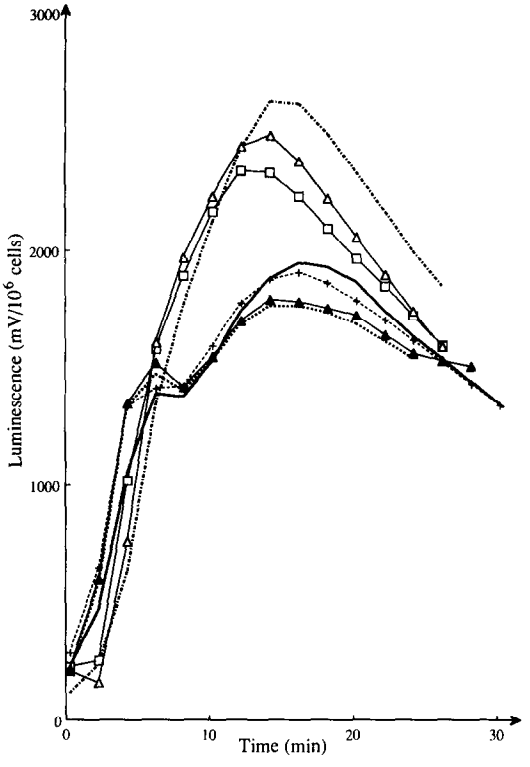

Fig. 3. Lucigenin-enhanced CL of PMA-stimulated PMNs previously incubated with mixtures of Leu-enkephalin and YGGYL. The concentrations used were: Leu-enkephalin 10^{-10} M (----); YGGYL 10^{-10} M (-·-·-); Leu-enkephalin 10^{-10} M + YGGYL 10^{-10} M (-+-+-); Leu-enkephalin 10^{-10} M + YGGYL 10^{-8} M (▲); Leu-enkephalin 10^{-9} M + YGGYL 10^{-10} M (□); Leu-enkephalin 10^{-8} M + YGGYL 10^{-10} M (△). The black line corresponds to the CL response of cells incubated without any peptide.

DISCUSSION

The diverse clinical sequelae of stress on the immune system may be due partly to the variety of central nervous system mediators released into the circulation. Kay *et al.* [15] have shown that the neuropeptide β-endorphin is released into the circulation during stress, and modulates human peripheral blood lymphocyte function. Enkephalin, β and γ-endorphin, and non-opioid derived fragments were shown to be potent at enhancing the human blood lymphocyte natural killer cell function with an inverted U-shaped dose–response relationship [15].

In the present study, lucigenin-enhanced CL assays were used in order to evaluate the effect of some opioid peptides and fragments, before and after

exposure to AOS, on the oxidative metabolism of human PMNs.

CL assays

Opioid peptides. Leu-enkephalin, β-endorphin and dynorphin were found to inhibit neutrophil respiratory burst induced by stimulation with PMA. The exposure of unstimulated cells to these peptides did not significantly affect PMN oxidative metabolism. Opioid peptide suppressive activity on PMA-induced respiratory burst was shown to disappear at peptide concentrations higher than 10^{-8} M and lower than 10^{-14} M, thus, yielding a U-shaped dose–response relationship, as observed previously by Diamant *et al.* [5] for pro-opiomelanocortine fragments.

Hydroxylated opioid peptides. The exposure of opioid peptides to AOS modified their structure by hydroxylation of phenylalanine residues, thus, producing hydroxylated derivatives [13]. Conversely to opioid peptides, the hydroxylated derivatives were found to enhance the PMA-induced respiratory burst of PMN at peptide concentrations higher than 10^{-14} M and lower than 10^{-8} M, giving rise to an inverted U-shaped dose–response relationship. The synthetic peptide YGGYL, used as a model of hydroxylated Leu-enkephalin, confirmed these results.

Non-opioid peptides. The non-opioid peptides tested FGGFL and GGFL which have no N-terminal tyrosyl residues, did not modify the CL response of human PMNs. Thus, tyrosyl residue seemed to be essential for inhibition of the PMN respiratory burst. After AOS treatment, FGGFL was converted into YGGYL and GGFL into GGYL. YGGYL only enhanced the CL response of PMA-stimulated PMNs. The absence of the N-terminal tyrosyl residue in GGYL probably abolished its effect on the CL response of PMA-stimulated PMNs. The importance of the N-terminal tyrosyl residue was confirmed in the time–response study that was carried out to evaluate the maximum incubation period necessary for Leu-enkephalin, after exposure to AOS, to exert its CL-enhancing activity (Table 1).

Thus, a reciprocal effect between opioid peptides and human PMNs could be postulated: AOS generated by PMA-activated PMNs hydroxylate opioid peptides which then increase the respiratory burst of PMNs. The question which arised was: did the opioid peptides and their corresponding hydroxylated derivatives compete for the same binding site?

A partial answer could be obtained by study of the effect of addition of high concentrations of Leu-enkephalin to PMNs incubated with low concentrations of YGGYL on the CL of PMA-stimulated PMNs, and of the effect of additon of high concentrations of YGGYL to PMNs incubated with low concentrations of Leu-enkephalin. Figure 3 shows that Leu-enkephalin and YGGYL binding sites are different since each of them could be saturated independently: high concentrations of YGGYL inhibited CL enhancement but not the CL-lowering caused by low concentrations of Leu-enkephalin whereas high concentrations of Leu-enkephalin remained without effect on the CL enhancement caused by YGGYL but inhibited the CL-lowering which was induced by low concentrations of Leu-enkephalin.

Further experiments are in progress to determine the exact origin of the observed CL enhancement in the presence of hydroxylated opioid peptides.

REFERENCES

1. Udenfriend S and Kilpatrick DL, Proenkephalin and the products of its processing: chemistry and biology. In: *The Peptides, Analysis, Synthesis, Biology* (Eds. Udenfriend S and Meienhofer J), Vol. 6, pp. 26–68. Academic Press, New York, 1984.
2. Hazum E, Ghang KJ and Cuatrecasas P, Specific non-opiate receptors for β-endorphin. *Science* **205**: 1033–1035, 1979.
3. McCain HV, Lamster IB, Bozzoni JM and Grbic JT, β-Endorphin modulates human immune activity via non-opiate receptor mechanisms. *Life Sci* **31**: 1619–1624, 1982.
4. Lopker A, Abood LG, Hoss W and Lionetti FJ, Stereoselective muscarinic acetylcholine and opiate receptors in human phagocytic leukocytes. *Biochem Pharmacol* **29**: 1361–1365, 1980.
5. Diamant M, Henricks PAJ, Nijkamp FP and De Wied D, β-Endorphin and related peptides suppress phorbol myristate acetate-induced respiratory burst in human polymorphonuclear leukocytes. *Life Sci* **45**: 1537–1545, 1989.
6. Ruff MR, Whal SM, Mergenhagen S and Pert CB, Opioid receptor mediated chemotaxis of human monocytes. *Neuropeptides* **5**: 363–365, 1985.
7. Van Epps DE and Saland L, β-Endorphin and met-enkephalin stimulate human peripheral blood mononuclear cell chemotaxis. *J Immunol* **132**: 3046–3051, 1984.
8. Smith EM, Harbour-McMenamin D and Blalock JE, Lymphocyte production of endorphin and endorphin-mediated immunoregulatory activity. *J Immunol* **135**: 779–782s, 1985.
9. Heijnen CJ, Kavelaars A and Ballieux RE, β-Endorphin: cytokine and neuropeptide. *Immunol Rev* **119**: 41–63, 1991.
10. Allen RC, Phagocytic leukocyte oxygenation activities and chemiluminescence. A kinetic approach to analysis. In: *Methods in Enzymology* (Eds. DeLucas MA and McElroy WD), Vol. 133, part B, pp. 449–493. Academic Press, London, 1986.
11. Samuni A, Black CDW, Krisna CM, Makech HL, Bernstein EF and Russo A, Hydroxyl radical production by stimulated neutrophils reappraised. *J Biol Chem* **263**: 13797–13801, 1988.
12. Brown SL, Tokuda S, Saland LC and Van Epps D, In: *Enkephalins and Endorphins. Stress and Immune Systems* (Eds. Plonikoff NP, Faith RE, Murgo AJ and Good RA), pp. 367–386. Plenum Press, New York, 1986.
13. Slaoui-Hasnaoui A, Guérin M-C and Torreilles J, Reciprocal effects between opioid peptides and human polymorphonuclear leukocytes—I. Chemical modifications of Leu-enkephalin by phorbol myristate acetate-stimulated polymorphonuclear leukocytes. *Biochem Pharmacol* **43**: 497–502, 1992.
14. Painter RG, Dukes R, Sullivan J, Carter R, Erdos EG and Johnson AR, Function of neutral endopeptidase on the cell membrane of human neutrophils. *J Biol Chem* **263**: 9456–9461, 1988.
15. Kay N, Morley JE and Van Ree JM, Enhancement of human lymphocyte natural killing function by non-opioid fragments of β-endorphin. *Life Sci* **40**: 1083–1087, 1987.