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Respiratory burst inhibition in human neutrophils by ultra-low doses of [D-Ala^2]methionine enkephalinamide

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An ultra-low dose (10^{-14} M) of opioid peptide [D-Ala^2]methionine enkephalinamide (DAMEA) is found to exert an inhibitory effect on the production of reactive oxygen species (respiratory burst) in human neutrophils. The validity of this phenomenon has been verified in a series of studies that comprised 30 experiments. The inhibition has proved to be statistically significant ($P<0.001$). The dose–response dependence of the effect (10^{-15}–10^{-9} M) followed a characteristic biphasic pattern (with the maximum effect at ultra-low doses). An opioid antagonist, naloxone partially blocks the inhibitory effect, which indicates that the DAMEA action is at least partially mediated by opioid receptors.

Ultra-low dose; Respiratory burst; [D-Ala^2]Met-enkephalinamide; Human neutrophil

1. INTRODUCTION

This paper deals with the effect of ultra-low doses of a stable methionine enkephalin analog. [D-Ala^2]-methionine enkephalinamide (DAMEA), on the production of reactive oxygen species (respiratory burst) in human neutrophils. The other types of opioid peptides, namely β-endorphin and dynorphin, were found to stimulate the respiratory burst in neutrophils at 10^{-12}–10^{-10} M [1]. In our experiments the inhibitory effect of DAMEA on the respiratory burst was observed at ultra-low doses, a phenomenon that has many question marks to it. Indeed, in spite of the recent communications on the effects by ultra-low doses (10^{-17}–10^{-14} M) of different agents on biological objects [2–4], many, if not the majority, of researchers consider these effects hardly probable because of unusually low number of acting molecules contained in an experimental volume (10^2–10^7 in 0.1–1 ml).

We have looked into the effect induced by ultra-low doses of DAMEA on the respiratory burst in human neutrophils (evoked by chemotactic peptide formyl-methionylleucylphenylalanine, fMLP). The inhibitory effect of 10^{-14} M DAMEA on the respiratory burst was proven to be statistically valid. Administration of naloxone, an opioid receptor antagonist, partially abolishes the inhibitory effect of enkephalin; this means that at least some part of the DAMEA action is mediated by specific opioid receptors.

Abbreviations: DAMEA, [D-Ala^2]methionine enkephalinamide; fMLP, formyl-methionylleucylphenylalanine; ROS, reactive oxygen species.

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2. MATERIALS AND METHODS

2.1. *Materials*

fMLP and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were from Sigma; DAMEA and naloxone were from Peptide Institute Inc. (Japan); HEPES was from Fluka; Dextran T-500 and Ficoll-Paque were obtained from Pharmacia.

2.2. *Solutions*

PBS: KH_2PO_4, 0.144 g/l, NaCl, 9 g/l, Na_2HPO_4, 0.795 g/l. HBSS, pH 7.4, with the addition of 20 mM HEPES, was prepared from a concentrated stock solution of HBSS (Sigma) without Phenol red and bicarbonate.

2.3. *Isolation of neutrophils*

The procedure was the same as described earlier [5]. The fraction of polymorphonuclear leukocytes (neutrophils) was kept on ice (4°C). A series of 10–15 experiments (luminescent studies) were started about 2 h after the adaptation of cells to post-isolation conditions.

2.4. *Luminescent studies*

The level of active oxygen generation by neutrophils was assayed by measuring the luminol-enhanced luminescence [6] in a luminometer LKB 1251. Luminol at a final concentration of 1 μM was placed into a luminometer polystyrene cuvette thermostatted at 37°C; DAMEA and (1–2)·10^6 cells (kept at 4°C prior to that) were added, and spontaneous chemiluminescence was recorded for 4–5 min; thereupon fMLP (10^{-7} M) was added. The response was recorded in mV. In each experiment simultaneous readings of 4–6 cuvettes were recorded, half of the number containing the control solution, and the other half the effector (DAMEA). Results were expressed as the ratio of mean chemiluminescence with DAMEA to that without.

2.5. *Data processing*

The results of experiments were processed using the Sigmaplot v.4.0 program (Jandel Scientific) on an IBM PC. Confidence intervals were determined by Student's t-test, the reliability of effect was estimated also by the non-parametric sign test.

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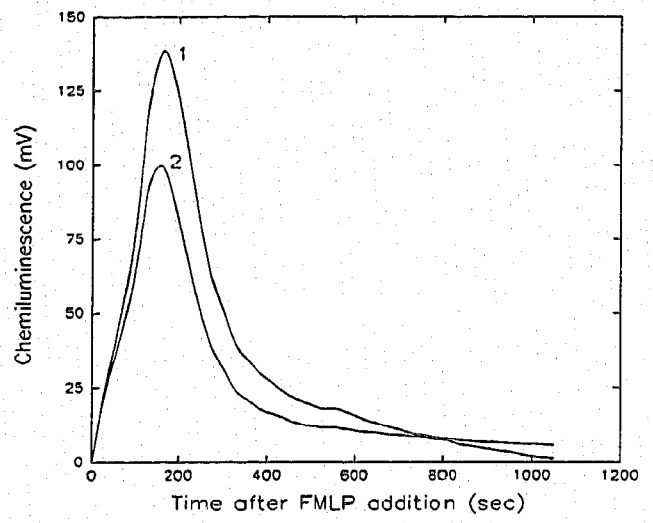

Fig. 1. Time course of the respiratory burst in human neutrophils (as luminol-enhanced luminescence) evoked by 10^{-7} M fMLP with (2) or without (1) addition of 10^{-14} M DAMEA.

3. RESULTS AND DISCUSSION

Typical curves showing the DAMEA effect on the kinetics of reactive oxygen species (ROS) generation (presented as luminol-enhanced luminescence) are represented in Fig. 1. The administration of enkephalin in a dose of 10^{-14} M results in a decrease both in the maximal rate of ROS generation and in the total amount of reactive oxygen generated (the integral below the curve). However, only a minor effect of 15–20% is observed.

There are some common regularities and problems in experiments with ultra-low doses. (1) Acting concentrations are 4–6 orders lower than dissociation constants

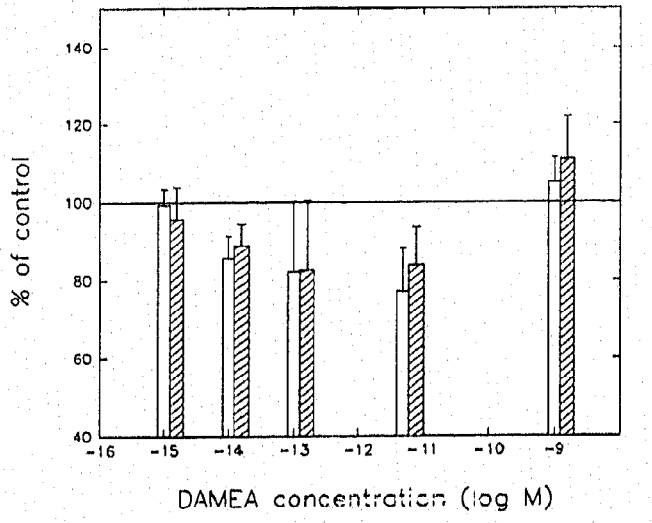

Fig. 2. Dose dependence of the DAMEA effect in % with respect to controls, mean values ±95% confidence intervals (30 replicates 10^{-14} M, 5 to 9 replicates for other concentrations). Left bars, the effect on the maximal rate of ROS generation; right bars, on the total amount of generated ROS.

K_d (which in our case is about 10^{-8} M [7]); this makes it difficult to explain the observed effects from a conventional standpoint. (2) The absence, as a rule, of the effects when usual (comparable to K_d, K_m) doses of the same compounds are used, or the presence of opposite effects at higher doses. (3) The poor reproduction of ultra-low dose effects, which does not make it possible to apply conventional criteria to validate the statistical significance of the phenomenon [2–4].

Therefore, we tested the validity of the phenomenon with special attention. The results indicate that 10^{-14} M DAMEA inhibits the maximal rate of ROS generation in 26 out of 30 experiments. The effect was observed in neutrophils of all blood donors (n=6). In some cases the effect value was as high as 40–55%. The inhibitory effect proved to be statistically significant according to both the sign test and Student's t-test (P<0.001).

A comparative analysis of the peptide effect on separate parts of the normalized kinetic curve (divided by the maximal response) enabled us to conclude that 10^{-14} M DAMEA also slightly decreases the rate of inactivation of ROS generation (displayed as the less steep decline at the descending part of curve 2 when compared to curve 1, Fig. 1). Although the observed effect is minor, it is significant according to the sign test (P<0.01, 30 replicates).

Studies on the DAMEA effect over a wide range of concentrations (Fig. 2) have shown that the dose–response dependence is of an 'inverse bell' shape, which is characteristic of the ultra-low dose effects described in the literature [2–4]. The respiratory burst inhibition is observed at 10^{-14}–10^{-11} M but absent at 10^{-15} and 10^{-9} M. There is a good correlation between DAMEA effects on the maximal rate of ROS generation and on the total amount of ROS generated during the experiment (left and right bars).

To test the specificity of DAMEA effects, we studied the action of DAMEA amino acids (tyrosine and methionine in particular), capable of comparatively simple oxidation–reduction transformations, and of the opioid antagonist naloxone on the respiratory burst. Neither tyrosine nor methionine (nor other DAMEA amino acids) in 10^{-14} M doses inhibit the respiratory burst in neutrophils (data not shown). Naloxone at 10^{-7} M partially blocks the 10^{-14} M DAMEA effect — the value of the DAMEA inhibitory effect in the presence of naloxone is 6.2±4.6% (mean ±95% confidence intervals) which is significantly (P<0.01) lower than the effect without naloxone (15.5±4.2%). Treatment with 10^{-7} M naloxone alone (without DAMEA) had no significant effect on respiratory burst (not shown).

The main conclusion that may be drawn from this experimental study is that the met-enkephalin analog DAMEA in a dose of 10^{-14} M inhibits the respiratory burst in human neutrophils, the effect being statistically significant with P<0.001. The inhibitory effect is not attributed to tyrosine or methionine, DAMEA molecule

amino acids being capable of oxidation–reduction reactions. Naloxone partially blocks the enkephalin inhibitory effect, which indicates that the DAMEA action is at least partially mediated by opioid receptors. Since this blockade is but partial (the DAMEA effect is still statistically significant, $P<0.05$), it is possible that human neutrophils also have naloxone-insensitive receptors for DAMEA. Another possibility, though less probable in our opinion, is that the peptide exerts a non-receptory effect on the respiratory burst.

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