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Inhibition of the respiratory burst in mouse macrophages by ultra-low doses of an opioid peptide is consistent with a possible adaptation mechanism

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Abstract The respiratory burst induced by phorbol myristate acetate in mouse macrophages was inhibited by ultra-low doses (10^{-15}–10^{-13} M) of an opioid peptide [D-Ala^2]methionine enkephalinamide. The effect disappeared at concentrations above and below this range. The inhibition approached 50% and was statistically significant ($P < 0.001$). Increasing the time of the opioid incubation with cells brought about a shift in the maximal effect to lower concentrations of the opioid (from 10^{-13} to $5 \cdot 10^{-15}$ M) and led to a decrease in the value of the effect, fully in accord with the previously proposed adaptation mechanism of the action of ultra-low doses.

Key words: Ultra-low dose; Kinetic mechanism; Opioid peptide; Macrophage; Respiratory burst

1. Introduction

The action of ultra-low doses (10^{-18}–10^{-14} M) of opioid peptides on cells of the immune system have recently been observed in several laboratories [1–5]. The effects of ultra low doses (ULD) of biologically active substances are unusual because the concentration wherein a response is observed is 4 to 6 orders lower than the lowest dissociation constants for ligand–receptor complexes (10^{-11}–10^{-10} M [6]). Another uncommon feature of the ULD effects is that they disappear with increasing the concentration of the acting substance (usually above 10^{-11}–10^{-9} M) [7,8]. In our recent paper [1] we have suggested a possible mechanism of the ULD action which provides an explanation for all the main features of the ULD effects. Among other things, the hypothesis makes a prediction that the concentration whereby a maximal effect is observed has to be shifted to lower values as the time of incubation of the active substance with target cells is increased [1].

To verify this prediction and to further establish the very existence of the effects of opioids in ULD we studied the action of an opioid peptide (DAMEA) on the respiratory burst in mouse peritoneal macrophages. The respiratory burst in mouse macrophages was significantly (up to about 50%) inhibited by ULD of DAMEA (10^{-15}–10^{-13} M). The inhibitory effect of DAMEA was abolished by naloxone suggesting that the DAMEA action was mediated by specific opioid receptors. With increasing the time of DAMEA incubation with cells from 10 to 30 min the maximal inhibitory effect on the dose dependence was shifted to lower concentrations (from about 10^{-13} M to about $5 \cdot 10^{-15}$ M) and its value decreased, fully in accord with the predictions of our hypothesis.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA) and luminol were from Sigma, naloxone and DAMEA were purchased from Peptide Institute Inc. (Japan), horseradish peroxidase was from Reakhim (Russian Federation). HBSS (pH 7.4) was prepared from a concentrated stock solution of HBSS (Sigma) without Phenol red and bicarbonate.

2.2. Isolation of macrophages

Peritoneal lavage from male mice of the CBA strain (18–20 g) was used as a source of resident peritoneal macrophages as described in [9]. The cells were resuspended in HBSS (at about $3 \cdot 10^6$ cells/ml) and kept on ice (40°C). Luminescent studies were started after about a 2 h adaptation of the cells to post-isolation conditions.

2.3. Luminescent studies

The level of active oxygen generation by the macrophages was assayed by measuring the luminol-enhanced luminescence [10] in an LKB 1251 luminometer. For further enhancing the response, horseradish peroxidase (10^{-11} M) was also added to the solution. At this concentration the peroxidase does not change the pattern of the respiratory burst and does not lead to self-activation of the burst (without added PMA), but enhances the response by about 300%. Luminol (10^{-6} M) and the peroxidase (10^{-11} M) were placed into a luminometer polystyrene cuvette thermostated at 37°C under constant stirring; about $3 \cdot 10^6$ cells (kept at 4°C prior to that) were added (final sample volume 1.2 ml) and spontaneous chemiluminescence was recorded for about 5 min; then DAMEA was added and the samples were incubated for 10 or 30 min. Thereafter a stimulus (PMA, 10^{-6} M) was added. The response was recorded in mV. In each experiment simultaneous readings from 4 cuvettes were noted, two of them having control samples and the other two containing also the added effector (DAMEA).

2.4. Data processing

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

3. Results and discussion

3.1. Inhibition of the respiratory burst at a constant time of incubation of cells with DAMEA

Typical examples of the time course of the respiratory burst in mouse macrophages (recorded as luminol-enhanced luminescence) induced by PMA in the presence and absence of DAMEA are presented in Fig. 1. A 10-min incubation with DAMEA in concentrations of 10^{-14}–10^{-13} M resulted in a decrease both in the maximal rate of ROS generation (the maxi-

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Abbreviations: DAMEA, [D-Ala^2]methionine enkephalinamide; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

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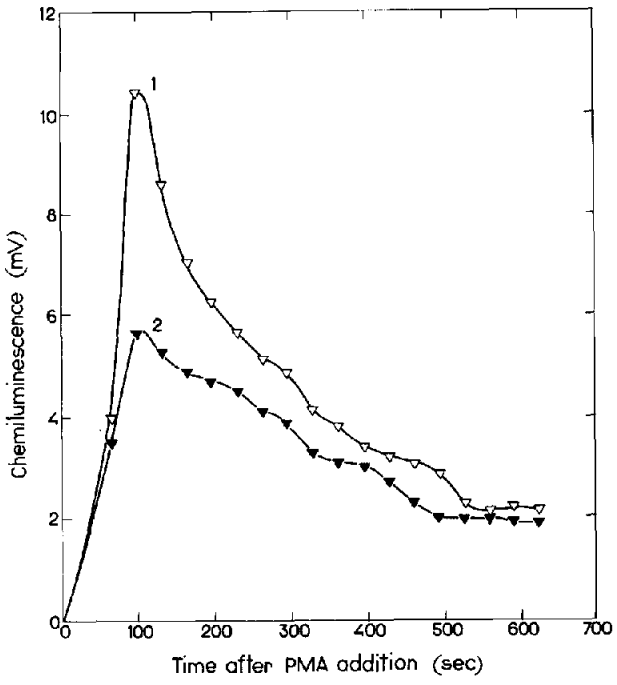

Fig. 1. The kinetic dependencies of the luminol-enhanced chemiluminescence (the respiratory burst) in mouse macrophages after adding the stimulus (PMA) at time $t = 0.1$ without DAMEA (1), and preincubated for 10 min with 10^{-14} M DAMEA (2).

mum on the curve) and in the total amount of reactive oxygen produced (the integral below the curve).

Taking into account the observed variance in the value of the effect particular attention was given to the verification of the results. For each concentration of the opioid the experiment was repeated 10^{-15} times. As can be seen in Fig. 2a, out of the studied DAMEA concentration range of 10^{-16}–10^{-7} M (10 min incubation with DAMEA) the inhibition (up to about 50%) occurred only at 10^{-14}–10^{-13} M. The effect disappeared at concentrations above and below this narrow range. The observed dose–response relationship is a bell-shaped dependence typical of the ULD effects. This relationship has also been found previously in our experiments with human neutrophils [1,5]. The effects were much the same for the maximal rate of ROS generation (Fig. 2a) and for the total amount of produced ROS (not shown). The inhibition by 10^{-14}–10^{-13} M DAMEA was statistically significant ($P < 0.001$).

3.2. *The adaptation mechanism of the action of opioid peptides and other biologically active substances in ultra-low doses*

In our recent paper [1] we proposed a possible mechanism of the ULD action. To gain a better understanding of the experimental results that follow, the hypothesis is briefly outlined below. We took, as the starting point for the hypothesis, the fact that the effects of ULD of a certain active substance often emerge in the presence of a much higher concentration of the same substance. In particular, such is the case for opioids which are presented at an endogenous concentration of 10^{-12}–10^{-10} M [1]. A cell is likely to adapt to the current concentration of the active substance and responds only to concentrational changes. Similar effects were observed in bacterial chemotaxis and interpreted by D.E. Koshland on the basis of the adaptation mechanism involving simultaneous fast activation and slow inhibition of a certain key process by the active compound [11]. A schematic representation of the model is given in Fig. 3. The ligand, L (active compound), acts on the transformations of factors (e.g. enzymes) E_1 and E_2 into their active forms (E^*_1, E^*_2), which have opposite effects (with different time constants) on the response, Z. From this scheme one can obtain the following equation for the dependence of the effect, Z, on the concentration of the added active compound, C, and the time of incubation with this compound, t [1]:

$$Z(t) = (L/(L+K_d) - L_0/(L_0+K_d)) \cdot (e^{-k_2(L+K_d)t} - e^{k_1(L+K_d)t}) \quad (1)$$

where L_0 is the initial concentration of the active compound, $L = L_0 + C$ is the concentration of the active compound after its addition, $K_d = k_{-1}/k_1$ is the dissociation constant for the interaction of the active compound with factor E_1.

This equation defines the bell-shaped dose–response dependencies typical for the ULD effects. From Eq. (1) it follows that as the time of incubation with the active compound increases the effect is shifted to lower concentrations and its value decreases, as shown in Fig. 4.

3.3. *The shift in the dose dependence of the DAMEA effect with increasing the time of incubation is consistent with the proposed adaptation mechanism*

To verify the above-mentioned prediction of our hypothesis we repeated the experiments presented in Fig. 2a increasing the time of incubation with DAMEA from 10 to 30 min (Fig. 2b).

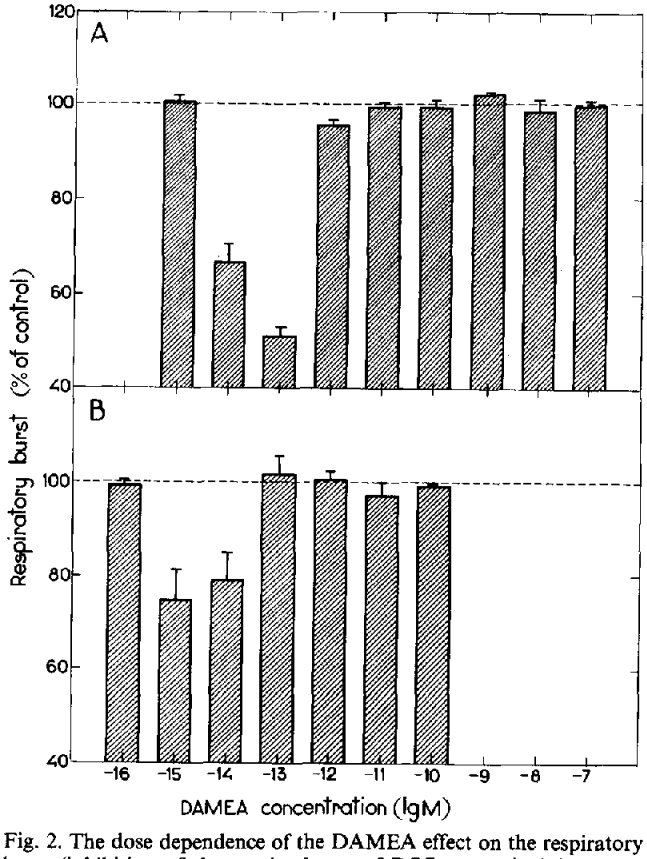

Fig. 2. The dose dependence of the DAMEA effect on the respiratory burst (inhibition of the maximal rate of ROS generation) in mouse macrophages. The bars represent mean ± S.E.M. from 10^{-15} replicates. The time of incubation with DAMEA: (A) 10 min, (B) 30 min.

Separate experiments revealed that incubation of cells without DAMEA at 37°C up to 30 min was without effect on the respiratory burst (not shown). As evident from Fig. 2a,b, the increased time of incubation with DAMEA led to a shift in the maximal effect to lower concentrations (from about 10^{-13} M to $5 \cdot 10^{-15}$ M) and to a decrease in the effect value (from about 50% to 25%). This result is fully consistent with our prediction (compare Figs. 2 and 4).

The antagonist of opioid receptors, naloxone, at 10^{-7} M abolishes the inhibitory effect of 10^{-14} M DAMEA (10 min incubation) on the respiratory burst: the effect decreases from 48 ± 7% to 6 ± 2% (mean ± S.E.M.) in the presence of naloxone. Treatment with naloxone alone had no significant effect on the respiratory burst (not shown). As we have reported previously, amino acids, constituting DAMEA, do not affect the respiratory burst in human neutrophils when added at 10^{-14} M [5]. These data indicate that the inhibitory effect of DAMEA is mediated by specific opioid receptors.

In principle, the exact mechanism of opioid action could be different from the simplest one given in Fig. 3. Particularly, in order to obtain theoretical dependencies (Fig. 4), which coincide very closely with the experimental ones (Fig. 2), we had to use a K_d value of 10^{-14} M, although for opioid receptors this value is about 10^{-10}–10^{-9} M [12]. Therefore in this case K_d in our model may represent an 'apparent' value which is due to some additional system of signal amplification.

In our view, the time-dependent shift shown in Fig. 2 provides good evidence for the proposed adaptation mechanism of the action of DAMEA in ULD, although it cannot be treated as a complete proof. Further consideration of the hypothesis requires us to determine the biochemical pathways involved in the reported process of inhibition by DAMEA, possibly with the use of appropriate specific inhibitors.

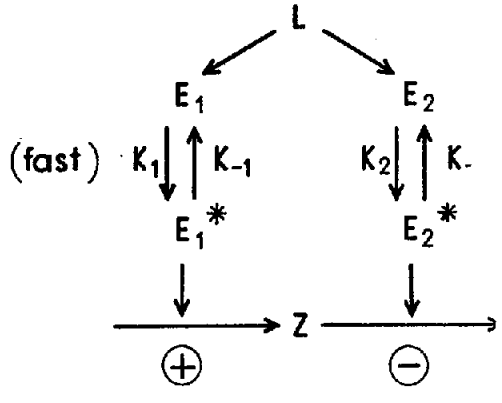

Fig. 3. The scheme of the adaptation model of the ULD action. L = the ligand (active compound), E and E* = the nonactive and active forms of the regulatory factors (e.g. enzymes), k = the rate constants, Z = the observed effect.

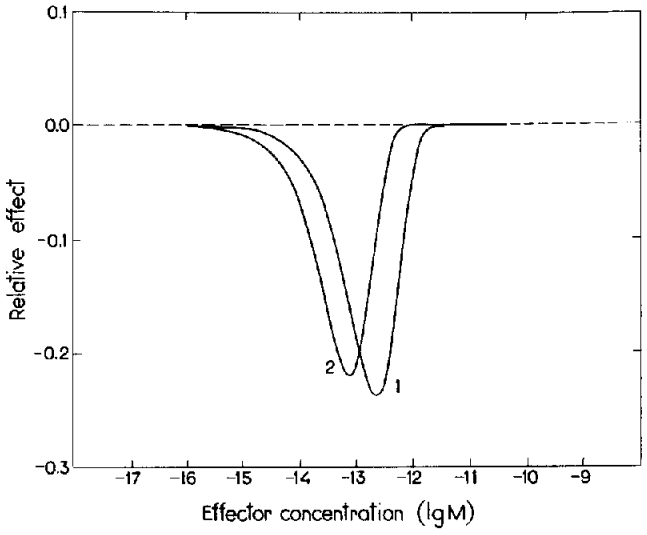

Fig. 4. Theoretical dose dependencies of the relative effect (Z) at different times of incubation (t) with an active compound. $L_0 = 0$, $k_1 = 10^{10}$ $M^{-1} \cdot s^{-1}$, $k_{-1} = 10^{-4} \cdot s^{-1}$, $k_1/k_2 = 2$. (1) t = 10 min, (2) t = 30 min.

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