STIMULATING ACTION OF ENDORHINS ON SYMPATHETIC GANGLION DEVELOPMENT IN CULTURE

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UDC 612.89 : 612.6.014.46 : [547.95 : 547.943]-085.23

KEY WORDS: endorphins, nerve tissue growth factors, culture.

Growth factors accelerating development and differentiation of nerve tissue cells are currently being intensively studied. Nerve growth factor (NGF) [13], glial growth factor [5], and several factors isolated from brain extract [4, 6, 12], are of special interest. The search for nerve growth substances among compounds playing an important role in function of the nervous system merit attention in this connection.

It has recenbly been shown that endogenous opioid peptides and their synthetic analogs accelerate healing of ulcers, due to their effect on properties of cells of the intestinal walls [3], i.e., structures where these compounds are widely represented normally [8] or, in other words, where these substances exhibit, not only their known analgesic properties, but also the properties of growth factors.

This paper describes an attempt to discover whether endorphins may have a role in changes in nerve tissue growth and differentiation.

EXPERIMENTAL METHOD

Tissue culture is the most adequate model with which to study growth properties of substances. Accordingly, an organotypical culture of sympathetic ganglia, which has been the commonest object used in investigations of this type [2], was used in the present case. Explants of the superior cervical ganglion of newborn Wistar rats were cultured on slides coated with collagen, in 35-mm Petri dishes (Falcon, USA) under standard conditions (37°C, 5% CO_2, humidity 98%), in growth medium of the following composition [1]: MEM medium (from Gibco, USA) 50%, embryonic calf serum 30%, Hanks' salt solution 20%, glucose 800 mg %, insulin 0.2 U/ml; γ- and β-endorphins, Leu- and Met-enkephalins were added to the medium in concentrations of $2 \cdot 10^{-18}$ to $2 \cdot 10^{-6}$ M. Commercial preparations of enkephalins (from Serva, West Germany) and peptides synthesized in the Laboratory of Peptide Synthesis by classical methods of peptide chemistry, were investigated and were characterized by values of specific rotation, results of amino-acid analysis, values of chromatographic mobility in various systems, and NMR spectra. NGF (from Collaborative Research, USA) was used in a concentration of $2 \cdot 10^{-8}$ M, and naloxone (from Endo Laboratories, USA) in concentrations of 10^{-7} to 10^{-4} M.

The cultures were grown in vitro for 2-3 weeks and the nutrient medium was changed twice a week. The cell composition and maximal size of the zone of growth were determined. The intensity of growth was estimated by a modified Tanaka's scheme [11]: the number of intersections of radially oriented axon-glial bundles in the zone of growth was counted in an assigned segment of 200 μ, at a distance of 250 μ from the edge of the explant, on the 3rd-4th day of culture. The state of the cultures was studied in preparations impregnations with silver by Holmes' method, and catacholamines also were demonstrated by a modified Lindvall's method. The results obtained in six series of experiments on 280 explants were subjected to statistical analysis by Student's test.

EXPERIMENTAL RESULTS

Growth and differentiation of sympathetic ganglia in culture medium of ordinary composition was of the standard character for the given conditions of culture (Fig. 1a) and corresponded to processes described in the

Laboratory of Cell Physiology and Laboratory of Peptide Synthesis, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR E. I. Chazov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 100, No. 8, pp. 242-245, August, 1985. Original article submitted December 26, 1984.

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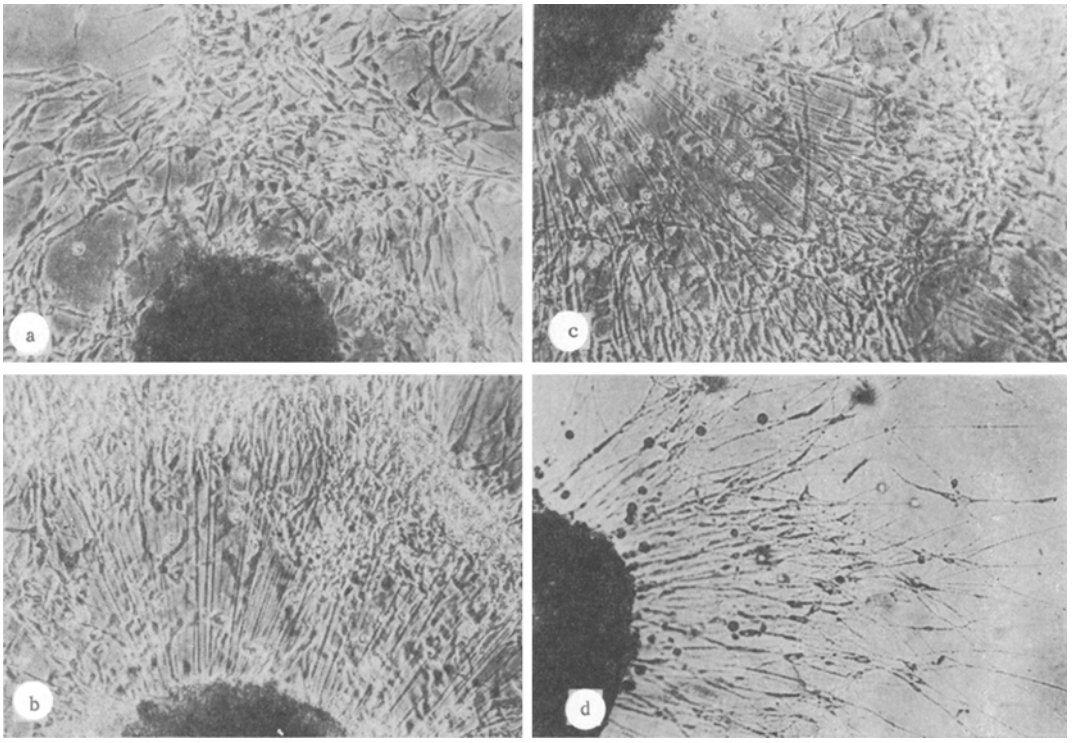

Fig. 1. Effect of endorphins on rat sympathetic ganglia (4 days in culture): a) control, b) Met-enkephalin ($2 \cdot 10^{-10}$ M), c) Leu-enkephalin ($2 \cdot 10^{-10}$ M), d) NGF ($2 \cdot 10^{-8}$ M). Intravital microscopy. Phase contrast. 250 ×.

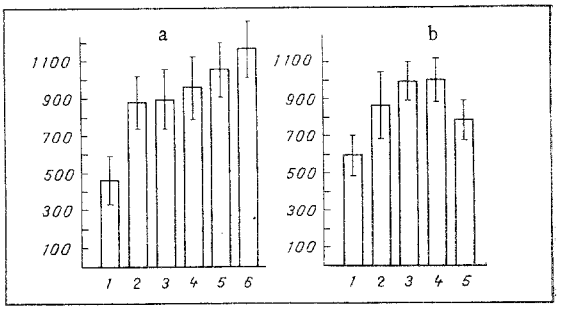

Fig. 2. Dependence of maximal size of zone of growth of explant on test peptides (a) and on concentration of Met-enkephalin (b). Abscissa: a) test substances: 1) control, 2) Leu-enkephalin, 3) Met-enkephalin, 4) γ-endorphin, 5) β-endorphin (in equimolar concentration of $2 \cdot 10^{-15}$ M), 6) NGF ($2 \cdot 10^{-8}$ M); b) concentration of Met-enkephalin: 1) control, 2-5) $2 \cdot 10^{-6}$, $2 \cdot 10^{-10}$, $2 \cdot 10^{-14}$, and $2 \cdot 10^{-18}$ respectively; ordinate, maximal size of zone of growth (in μ).

literature [2]. The explants developed at a moderate rate. By the 3rd-4th day in culture there were few axons in the zone of growth and their distribution around the circumference of the explant was uneven. Only migration of fibroblast-like cells and glia into the zone of growth and moderate proliferation of these cells were observed. During further culture for 2-3 days a zone of growth gradually formed and was composed mainly of nonneural elements. The number of axons in the zone of growth decreased, as shown by a decrease in the number of catecholaminergic fibers outside the explant. The largest size of the zone of growth of sympathetic ganglia by the 3rd-4th day of culture under normal conditions varied from 300 to 500 μ. The number of axon-glial bundles did not exceed 8-10 in a 200-μ segment.

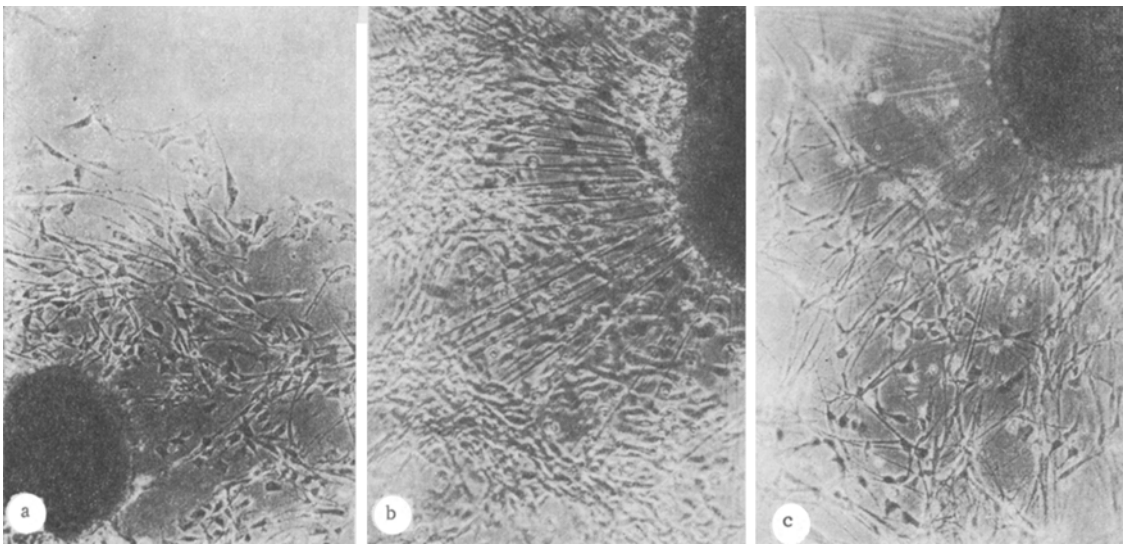

Fig. 3. Effect of naloxone on rat sympathetic ganglia (3rd day in culture): a) control, b) naloxone (10^{-6} M) and Met-enkephalin ($2 \cdot 10^{-8}$ M), c) naloxone (10^{-6} M). Intravital microscopy. Phase contrast. 250 ×.

Addition of endorphins in optimal concentrations (10^{-8}–10^{-12} M) led to sharp changes in the composition and size of the zone of growth: γ- and β-endorphins and Leu- and Met-enkephalins appreciably increased the number of glial and fibrolast-like cells in the zone of growth. By the 2nd-3rd day of culture clearly formed, radially oriented axon-glial bundles could be seen. They formed a network around the explant, fringed by a "halo" of widely migrating (by more than 700 μ) glial and fibroblast-like cells (Fig. 1b, c). The zone of growth varied in size from 800 to 1300 μ (Fig. 2a) and the number of axon-glial bundles reached 30 to 40 per 200-μ segment. Incidentally, commercial preparations of Leu- and Met-enkephalins (from Serva, West Germany), which were used in some experiments, gave a similar effect.

Comparison of the stimulating action of endorphins with the effect of NGF, used as the standard (Fig. 1d), showed that in the latter case the zone of growth was a little larger (Fig. 2a, b) and more "translucent" (Fig. 1d), probably evidence that NGF has no effect on glial and fibroblast-like cells.

The action of the four test peptides was subjected to comparative analysis in a series of experiments in which they were used in an equimolar mean-effective concentration under our conditions (Fig. 2a). The maximal size of the zone of growth for all agents in a concentration of $2 \cdot 10^{-10}$ M was found to be about twice its size in the control ($464 \pm 127\,\mu$), and was $879 \pm 136\,\mu$ for Leu-enkephalin, $900 \pm 160\,\mu$ for Met-enkephalin, $959 \pm 170\,\mu$ for γ-endorphin, and $1056 \pm 137\,\mu$ for β-endorphin. Under the influence of NGF the zone of growth of $1150 \pm 148\,\mu$.

Dependence of the magnitude of the response on the dose of peptide was studied in the case of Met-enkephalin, using concentration of $2 \cdot 10^{-18}$ to $2 \cdot 10^{-6}$ M (Fig. 2). Over a wide range of concentrations (from 10^{-10} to 10^{-14} M) the magnitude of the response differed statistically from the control ($P \leq 0.05$). With higher (10^{-6} M) and lower (10^{-18} M) peptide concentrations only a weak tendency (not significant) was observed for the zone of growth to enlarge compared with its normal size.

The dose–effect dependence is thus reflected by a curve with a maximum in the range of very low concentrations; this probably points to a possible receptor mechanism of the growth-stimulating action of neuropeptides on nerve tissue cells.

To study to what extent the action of peptides on growth and differentiation of nerve tissue is connected with opiate receptors of nerve cells, experiments were carried out with naloxone, a specific blocker of these receptors. These experiments showed that naloxone, if added to the growth medium together with the test paptides, did not depress their nerve growth effect: There was no decrease in the number of catecholaminergic fibers in the zone of growth. Only a small decrease in the response of the glia and fibroblast-like cells was observed (Fig. 3b). It was also found that naloxone itself, over a wide range of concentrations (from 10^{-7} to 10^{-4} M) has a stimulating action on growth of sympathetic ganglia in culture (Fig. 3c). In all concentrations

studied naloxone caused moderate stimulation of growth of axons and an increase in the number of nonneural cells in the zone of growth by the 2nd-3rd day of culture.

It can accordingly be concluded from the results of this investigation that opioid neuropeptides have a sufficiently marked growth-promoting action on nerve tissue structures in culture. The effect of these substances is twofold: first, they cause changes in differentiation of the neurons and stimulate growth of axons, which is probably comparable with the effect of NGF [13], and second, they cause changes in the number of glial and fibroblast-like cells in the zone of growth, and may thus be comparable with glial growth factor [5]. Further analysis of the effect of endorphins on growth and functional properties of nerve tissue cells in culture will be undertaken by the use of a radioisotope technique and analysis of changes in the rate of migration, so that it will be possible to differentiate between proliferation and migration.

The unusually low concentrations of peptides (10^{-10}-10^{-14} M) exerting a growth-stimulating effect are interesting. Results of experiments to study receptor binding of opiates and their antagonists reveal a limiting value of 10^{-10} M [8, 14], two orders of magnitude higher than the effective concentration established by our own experiments. It can be tentatively suggested that the sensitivity of nerve tissue to endorphins, which play the role of regulators of growth and differentiation, is significantly greater than in cases when these compounds exhibit the properties of analgesics.

The absence of a blocking effect of naloxone, noted in this investigation, does not rule out the probability that opioid peptides act through a receptor mechanism, for there are indications that naloxone does not abolish the effect of opiate peptides in all [3, 7, 10].

From the analogy with factors isolated from brain extract [4, 12] which, as we know, increase the rate of differentiation of neurons and glia, and also enhance their viability, it can be concluded that the group of endogenous substances (opioid and neuropeptide) possesses the properties of nonspecific nerve tissue growth factors. Recently published data [15], indicating a definite role of opioid peptides in regulation of development of the nervous system, may be confirmation of this view.

The distribution of endogenous opiates in the central and peripheral nervous system suggests that endorphins may play a definite role in nerve tissue regeneration. However, this hypothesis requires further experimental investigation.

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