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Opioid peptides inhibit the estradiol-induced proliferation of cultured rat uterine cells

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Abstract

The effect of opioid peptides on estradiol-induced cell proliferation in adult rat uterine primary cell cultures was studied. Estradiol increased cell density by 40%. This estradiol-induced stimulation of cell proliferation was decreased to control values by [D-Met^2,Pro^5]enkephalinamide. The opioid-induced inhibition of uterine cell proliferation was blocked completely by the specific opiate antagonist naloxone, while naloxone did not have any effect on its own. The inhibition of cell proliferation by enkephalinamide was apparent at each stimulatory estradiol concentration examined. This opioid effect was mediated mainly by the μ opiate receptor. The observed effects occurred within the physiological nanomolar concentration range. Enkephalinamide did not have any effect on the basal proliferation rate of adult rat uterine cells. However, enkephalinamide inhibited the basal rate of cell proliferation in cell cultures prepared from 7-day-old immature rats. In summary, here we present evidence of novel physiological direct cross-talk between the opioid and estrogenic signaling systems in the regulation of normal uterine growth. © 1997 Elsevier Science B.V.

Keywords: Opioid; Estradiol; Uterus; Proliferation; Cell culture; (Rat)

1. Introduction

There is a growing amount of evidence on the inhibitory role of endogenous opioid peptides in the regulation of cell proliferation. However, the functional physiological significance of this is still debated. The inhibitory role of endogenous opioid peptides in cell proliferation has been described in the central nervous system (Vértes et al., 1982; Zagon and McLaughlin, 1988, 1991) and in some peripheral organs and tissues (Zagon et al., 1994; Radulovic et al., 1995), including their malignant forms (Zagon and McLaughlin, 1988; Hytrek et al., 1996b). The direct effect of opioid peptides at the cellular level has been demonstrated in neuronal (Davila-Garcia and Azmitia, 1990), glial (Barg et al., 1994), and spinal-cord ganglion (Barg et al., 1993) cultures.

The endogenous opioid peptides (Wahlström et al., 1985; Li et al., 1991), as well as opioid receptors (Baraldi et al., 1985; Vértes et al., 1986, 1993) and their mRNA messages (Jin et al., 1988; Muffly et al., 1988; Low et al., 1989; Wittert et al., 1996), are present in the female reproductive tract and are regulated by ovarian steroids (Baraldi et al., 1985; Petraglia et al., 1985; Wahlström et al., 1985; Vértes et al., 1986; Jin et al., 1988; Low et al., 1989). However, data on the possible direct cell proliferation-inhibitory action of endogenous opioid peptides in vitro in female reproductive tissues are available for neoplastic established human breast cancer cell lines only (Maneckjee et al., 1990; Hatzoglou et al., 1996). These data show the role of opioid peptides in carcinogenesis but do not provide direct clues to their role in physiological regulation.

Previously we described that endogenous opioid peptides inhibit cell proliferation in vivo in the adult (Ördög et al., 1992, 1993) and developing (Vértes et al., 1995b, 1996) rat uterus. In this study, we wanted to obtain in vitro data on the possible direct mechanism of action of endogenous opioid peptides in normal adult rat uterine cells. Here we present novel evidence for a direct action of endogenous opioid peptides in cultured normal rat uterine cells, which suggests that these peptides have a possible physiological role.

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2. Methods

2.1. Animals

Adult cycling female rats from the CFY strain (15–20 per experiment) were ovariectomized, then pretreated with estradiol (10 μg/g.b.w.) by intraperitoneal injection on the 7th and 8th day following ovariectomy at 9 a.m. and 4 p.m. The next day animals were decapitated and uteri were removed in a sterile way. Uteri were also obtained from 7-day-old immature female rats that had not been treated with estradiol.

2.2. Materials

The following chemicals were purchased from Sigma: Hank's Balanced Salt Solution with calcium ions and magnesium ions (HBSS-2), Hank's Balanced Salt Solution containing no calcium ions and no magnesium ions (HBSS-0), Trypsin-EDTA, HEPES, Dulbecco's Modified Eagle's Medium (DMEM), antibiotic–antimycotic liquid, Kanamycin, estradiol-17β, naloxone, [D-Ala^2,*N*-Me–Phe^4,Gly^5-ol]enkephalin (DAMGO), [D-Pen^{2,5}]enkephalin (DPDPE) and porcine Dynorphin-A. [D-Met^2,Pro^5]enkephalinamide was a generous gift from Dr. S. Bajusz (Institute for Drug Research, Budapest, Hungary). Fetal bovine serum was purchased from Protein (Gödöllö, Hungary). Each procedure was performed at 4°C unless otherwise stated.

2.3. Establishment of uterine primary cell cultures

To obtain rat uterine primary mixed-cell cultures we used an economic modification of a published method (McCormack and Glasser, 1980; Markaverich et al., 1981). Uteri were cleaned free from debris and fat, the horns were opened and tissues were minced into 1–2 mm pieces, using two scalpels. Uterine cells were dispersed by limited enzymatic digestion with HBSS-0 solution containing 0.125% Trypsin-EDTA, 2% HEPES, pH 7.4. Two 50 ml centrifuge tubes contained not more than 1.5 g tissue each in 10 ml Trypsin-EDTA. The first incubation was performed at 4°C for 1 h, and then tubes were transferred to 22°C for 1 h. Vortexing was done regularly every 5 min. At the end of this incubation the sample was filtered through a cell dissociation sieve (mesh 60 μm) followed by three washes with 10 ml of HBSS-2 containing 1% fetal bovine serum, and then the remaining tissue was placed back into the incubation tubes in 10 ml fresh Trypsin-EDTA. The resulting cell suspension was centrifuged at $300 \times g$, and the pellet was resuspended in HBSS-2 and kept on ice until all fractions were obtained. The second incubation was performed at 37°C for 15 min, and third and fourth incubations were at 37°C for 30 min. The four cell pellets were combined and washed three times with HBSS-2, and then resuspended in culture medium (see below) to reach a final 10 000 viable cells (larger than 20 μm diameter) per cm^2 plating density in the 25 cm^2 Corning culture flasks, yielding approximately 1000 attached cells per cm^2 in one day. A similar method was used for the experiments with cells from 7-day-old rats except that one incubation with Trypsin-EDTA solution (37°C, 15 min) was enough to disperse the uterine cells completely.

2.4. Culture medium and treatments

Cells were cultured in DMEM containing 10% fetal bovine serum, 2% antibiotic–antimycotic liquid, 0.4% Kanamycin, 2% HEPES pH 7.4. Experiments were performed in the primary culture phase after a two-day initial attachment period. Each test agent was added simultaneously to the culture medium in the morning of the 3rd day and was present during the entire experimental culture period for a maximum of 10 days. Culture media containing the corresponding agent(s) were changed at 48 h intervals.

2.5. Determination of cell densities

In the 10–12-day-old subconfluent cultures, following trypsinization of the monolayers, cells were counted in hemocytometers by two independent investigators who were without knowledge of the other's results. Occasionally parallel counting was performed in an automatic cell counter (Coulter Counter ZM) apparatus as well, giving similar results.

2.6. Statistical analyses

Figures are representatives of at least three experiments giving similar results. Data are expressed in 10^3 cells/cm^2 units, showing the means and their standard errors of six data points from one representative experiment. Analysis of variance followed by post hoc Student–Newman–Keuls multiple range test (Dowdy and Wearden, 1983) was used for data analysis.

3. Results

Rat uterine primary cell cultures were established successfully. The yield was approximately 10 million viable cells from 20 rat uteri. With an average population doubling time of 1.5–2.4 days during the log phase of culture, monolayers reached confluence after 10–14 days and contained roughly equal numbers of epithelioid and myofibroblast-like cells. The characteristics of each cell type present in our primary rat uterine cultures were determined morphologically on photographs of cells viewed through a phase contrast microscope (not shown). Large, flat cells closely attached to each other, thus forming compact

groups, were considered as transformed blast type normal epithelioid cells. The elongated individual cells that spread and moved around were considered to be myofibroblasts. They were organized in parallel rows in confluent monolayers. Following a 2–3-day latency period, 2.2 nM estradiol resulted in a 40% increase of cell density (Fig. 1). This stimulation of cell proliferation was completely eliminated by the presence of 30 nM enkephalinamide, after which cell density was close to the corresponding control values (Fig. 1). Treatments did not affect the relative amount of epithelioid and myofibroblast-like cells in the monolayers. Mixed cell primary cultures were used because full scale estrogenic action needs the presence of stromal and epithelial and/or myometrial smooth muscle cells (Astrahantseff and Morris, 1994). As the fetal bovine serum present in our culture medium might contain estrogenic hormones and might bias our observed proliferative effects, we used charcoal stripped FBS in some parallel early experiments with no change in the final results.

The concentration dependence of the enkephalinamide effect (Fig. 2) revealed that the half effective inhibitory concentration of enkephalinamide to antagonize the stimulation of cell proliferation by estradiol was about 3 nM. No effect could be observed below 1 nM and complete inhibition was reached at 100 nM and above (Fig. 2).

The inhibitory effect of 30 nM enkephalinamide was abolished by concomitant administration of 30 nM naloxone (Fig. 3). Naloxone alone did not have any effect on either basal or estradiol-induced cell proliferation. The basal cell proliferation was not affected by the presence of enkephalinamide (Fig. 3).

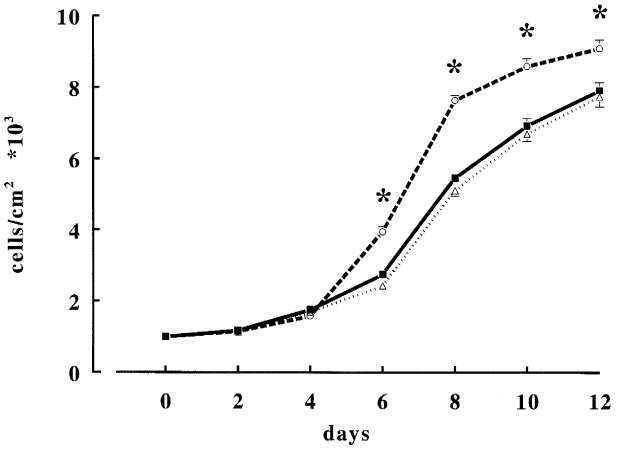

Fig. 1. Time dependence of the inhibitory effect of [D-Met²Pro⁵]enkephalinamide on the estradiol-induced stimulation of cell proliferation in rat uterine primary mixed-cell cultures. Agents were added to the culture medium after an initial 2-day attachment period. Open circle with dashed line: estradiol 2.2 nM, filled square with solid line: estradiol 2.2 nM and enkephalinamide 30 nM, open triangle with dotted line: control, no treatment. Analysis of variance: $F = 0.06, 2.04, 38.12, 84.96, 23.63, 6.66$ for the 2, 4, 6, 8, 10 and 12 day data sets, respectively. Post hoc Student–Newman–Keul's multiple range tests: values marked by * are significantly different from the corresponding control values at $P < 0.01$ level.

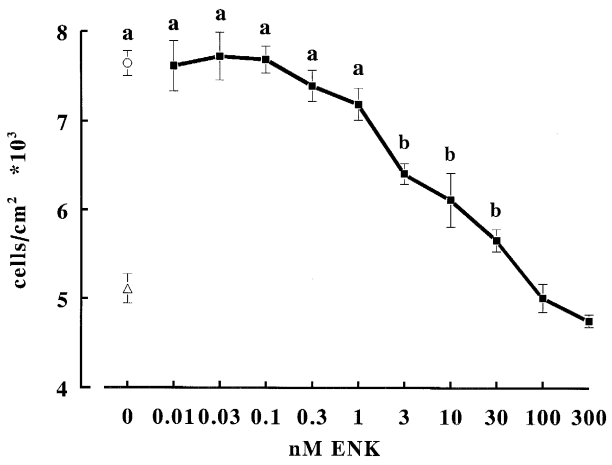

Fig. 2. Concentration dependence of the inhibitory effect of enkephalinamide (ENK). Filled squares: estradiol 2.2 nM and enkephalinamide 0.01–300 nM, open circle: estradiol 2.2 nM alone and open triangle: control, no treatment. Analysis of variance: $F = 37.35$, post hoc Student–Newman–Keuls multiple range test: values marked by different letters (a, b) are significantly different from control and from each other at $P < 0.01$ level.

The effect of selective opioid peptides was also studied. DAMGO, DPDPE and Dynorphin-A (100 nM each) were added to the culture medium (Fig. 4). A complete inhibitory effect on estradiol-induced stimulation of cell proliferation by DAMGO was observed, while Dynorphin-A had a marginal effect and DPDPE did not change cell density at all. These opioid peptides, like enkephalinamide, did not have any effect on basal cell proliferation (Fig. 4).

We examined the inhibitory effect of enkephalinamide at various estradiol concentrations (Fig. 5). The concentration dependence curve of estradiol revealed that the cell density of rat uterine primary mixed-cell cultures was

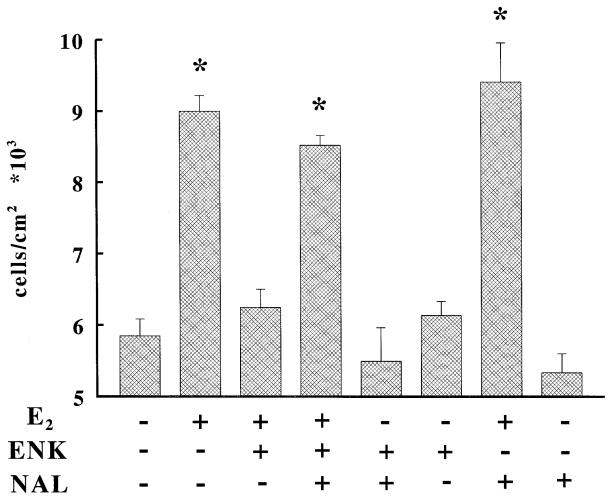

Fig. 3. Blockade by Naloxone (NAL) of the inhibitory effect of enkephalinamide (ENK). Cross marks: estradiol (E_2) 2.2 nM, enkephalinamide 30 nM and naloxone 30 nM were present. Analysis of variance: $F = 23.37$, post hoc Student–Newman–Keuls multiple range test: values marked by * are significantly different from control at $P < 0.01$ level.

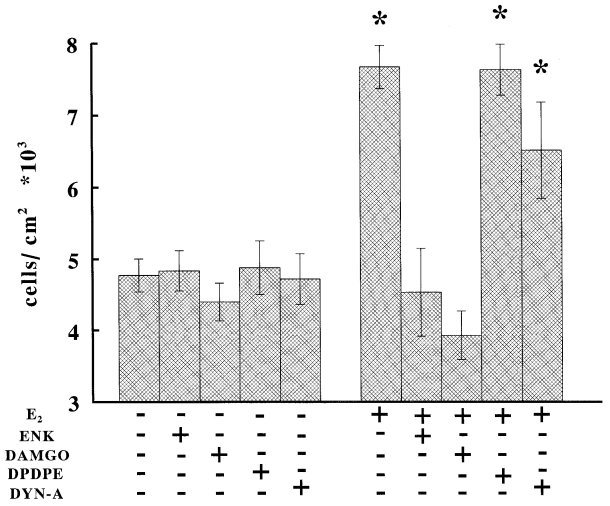

Fig. 4. Opiate receptor subtypes involved in the inhibitory effect of enkephalinamide (ENK) on estradiol-induced (E_2) stimulation of cell proliferation in rat uterine primary mixed-cell cultures. Cross marks: estradiol 2.2 nM, enkephalinamide 100 nM, DAMGO 100 nM (μ selective), DPDPE 100 nM (δ selective) and Dynorphin-A 100 nM (κ selective) were present. Analysis of variance: $F = 11.59$, post hoc Student–Newman–Keuls multiple range test: values marked by * are significantly different from control at $P < 0.01$ level.

increased by estradiol over the physiological 0.1–3 nM range. Estradiol had no effect below 0.1 nM, showed a diminished effect at 3 nM and did not stimulate cell growth at or above 10 nM (Fig. 5). The presence of enkephalinamide in the culture medium completely blocked the stimulatory effect of estradiol at all estradiol concentrations examined (Fig. 5).

The results of experiments with cultured uterine cells prepared from immature 7-day-old rats (Fig. 6) showed that, similar to results obtained in vivo (Vértes et al., 1995b), endogenous opioid peptides inhibited the basal cell

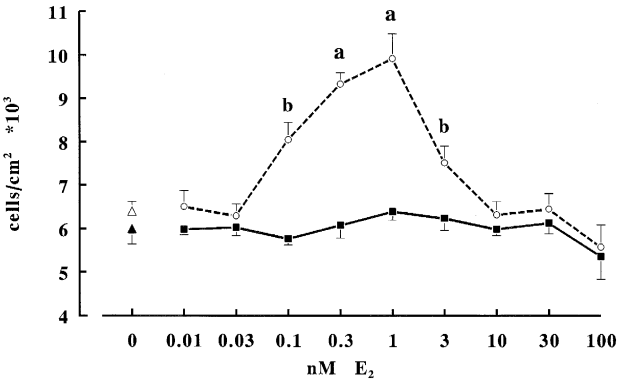

Fig. 5. Concentration dependence of the stimulatory effect of estradiol (E_2) on cell proliferation in rat uterine primary mixed-cell cultures and its inhibition by enkephalinamide. Open circle: estradiol 0.01–100 nM, filled square: estradiol 0.01–100 nM and enkephalinamide 100 nM, filled triangle: enkephalinamide 100 nM alone, and open triangle: control, no treatment. Analysis of variance: $F = 13.33$, post hoc Student–Newman–Keuls multiple range test: values marked by different letters (a, b) are significantly different from control and from each other at $P < 0.01$ level.

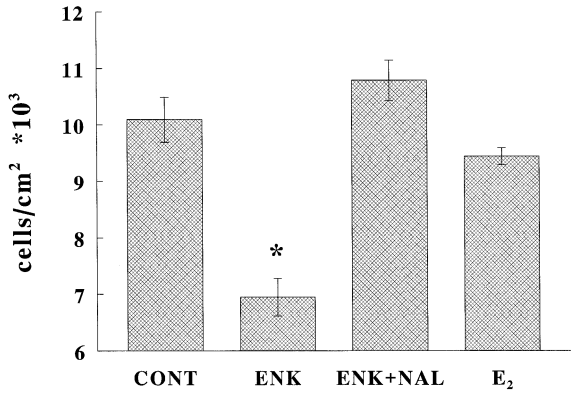

Fig. 6. Inhibitory effect of enkephalinamide (ENK) on basal cell proliferation in rat uterine primary mixed-cell cultures prepared from 7-day-old immature rats. Enkephalinamide 100 nM, naloxone (NAL) 100 nM and estradiol (E_2) 2.2 nM were present in the medium. Analysis of variances: $F = 26.68$, post hoc Student–Newman–Keuls multiple range test: * marks value significantly different from control at $P < 0.01$ level.

proliferation of cultured uterine cells in vitro. This inhibitory effect of enkephalinamide was eliminated by naloxone (Fig. 6), while estradiol was unable to stimulate cell proliferation in these cultures.

4. Discussion

Ample evidence has accumulated for the important role of endogenous opioid peptides in the regulation of hypothalamic GnRH and pituitary gonadotroph hormone output (Ferin et al., 1984; Genazzani and Petraglia, 1989). We described recently the in vivo inhibitory effect of endogenous opioid peptides on estradiol-induced DNA synthesis in adult (Ördög et al., 1992, 1993) and on basal DNA synthesis in developing (Vértes et al., 1995b, 1996) rat uterus. As gonadotropins can directly regulate uterine cell proliferation (Környei et al., 1993, 1996; Mukherjee et al., 1994), our previous in vivo studies could not rule out the possibility that endogenous opioid peptides might exert their observed cell proliferation inhibitory effect via other endocrinological substances, thus acting indirectly on normal uterine tissues.

In our present experiments we showed that endogenous opioid peptides can inhibit the estradiol-induced cell proliferation of cultured rat normal uterine cells in a time- and concentration-dependent manner, suggesting a direct mechanism of action at the cellular level. The effective concentrations were within the physiological range and naloxone could completely eliminate the inhibitory effect, suggesting a receptor-mediated mechanism of action. Our novel results are consistent with previously published data showing that the estradiol-inducible fraction of cell proliferation in adult reproductive tissues can be inhibited by endogenous opioid peptides, while basal proliferation is not affected (Maneckjee et al., 1990; Abou-Issa and Tejwani, 1991; Ördög et al., 1992; Vértes et al., 1996).

The receptor subtypes involved were found to belong mainly to the μ and to a lesser extent to the κ opiate receptor subtypes. These findings are consistent with the presence of mainly μ, and to a lesser extent δ and κ, receptor mRNA in rat uterus (Wittert et al., 1996) and with the μ-preferring binding characteristics of enkephalinamide (Rónai et al., 1981). However, there is a partial contradiction with our previous findings in rat uterine in vivo experiments, where δ receptor involvement was shown to be predominant (Ördög et al., 1992). The in vivo administration, differences in the degradation rates of specific peptides, and the great differences in the duration of exposure to endogenous opioid peptides might be responsible for this discrepancy.

In our estradiol dose dependence experiments, estradiol stimulated rat uterine cell proliferation in the physiological nanomolar range. The observed decrease in the stimulatory ability of estradiol at and above 3 nM may also be consistent with physiological regulation, as the well-known hypothalamic negative to positive estradiol feedback mechanism works over the same critical concentration range, i.e.: 2 nM and above. In agreement with data in the literature (Nardulli and Katzenellenbogen, 1986; Borras et al., 1994), we suppose that the long-lasting presence of high concentrations of estradiol in the medium might cause some form of down-regulation of one or more part(s) in the mechanism of action of estradiol. Enkephalinamide was able to inhibit completely the stimulation of cell proliferation at all effective estradiol concentrations examined. The estradiol-induced fraction of cell proliferation has been described to be inhibited by endogenous opioid peptides in the adult rat uterus (Ördög et al., 1992, 1993; Vértes et al., 1996) in vivo and in some neoplastic established human breast cancer cell lines (Maneckjee et al., 1990; Hatzoglou et al., 1996) in vitro. The basal cell proliferation was not affected. The interaction between estradiol and endogenous opioid peptide systems can even be observed at the receptor level. In our previous binding studies endogenous opioid peptides and estradiol could even displace each other from the type II estradiol binding sites that are closely related to true uterine growth (Garai et al., 1989). Opioid peptides exert their action by activating their cell membrane receptors. However, the additional intracellular localization of functional opiate receptors (Garai et al., 1989; Szücs et al., 1990; Belcheva et al., 1993; Vértes et al., 1995a; Hytrek et al., 1996b) makes even intracellular cross-talk possible.

Endogenous opioid peptides inhibit basal cell proliferation even in the absence of estradiol in developing rat uterus both in vivo (Vértes et al., 1995b, 1996) and in vitro (see above) and in various peripheral normal and tumorous tissues and cells (Zagon et al., 1994, 1996; Hatzoglou et al., 1996; Hytrek et al., 1996a). As cell proliferation is considered to be stimulated mainly by growth factors in these cases, endogenous opioid peptides might act directly on the growth factor systems as well.

On the basis of our present and previously published results (Ördög et al., 1992, 1993; Vértes et al., 1995b, 1996), it can be concluded that the inhibitory effect of opioids on cell proliferation in the uterus is dependent on age and on circulating estradiol levels. The tonic inhibition by endogenous opioid peptides of intensive basal cell proliferation, possibly driven by growth factors, in younger immature animals turns into inhibition of estradiol-induced cell proliferation in adult animals, when the responsiveness of uterine cells to estradiol is fully developed.

Clarification of the detailed mechanism of action of the novel cross-talk between the opioid and the estrogenic and/or growth factor signaling systems in uterine physiology requires further experiments.

Taken together, here we show for the first time that the estradiol-induced cell proliferation of cultured normal rat uterine cells can be inhibited by opioid peptides in vitro. This opioid effect is mediated by a direct receptor mechanism of action, mainly involving the μ opiate receptor subtype. The observed effects occurred within the physiological (nanomolar) concentration range of opioid peptides. Based on our results, a novel physiological inhibitory role of endogenous opioid peptides could be suggested in the regulation of normal uterine growth, with the peptides having a direct local mechanism of action.

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