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Short communication

Regulation of activator protein-1-DNA binding activity by opioid peptides in estrogen-sensitive cells of rat hypothalamus and uterus

Angéla Oszter [a,*], Beáta Törőcsik [b], Zsuzsanna Vértes [a], József L. Környei [a], Kálmán A. Kovács [c], Marietta Vértes [a]

[a] *Institute of Physiology, University Medical School of Pécs, Pécs, Szigeti str.12, H-7643 Hungary*
[b] *Department of Biology, University Medical School of Pécs, Pécs, Szigeti str.12, H-7643 Hungary*
[c] *Department of Obstetrics and Gynecology, University Medical School of Pécs, Pécs, Szigeti str.12, H-7643 Hungary*

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Abstract

The present studies demonstrate, for the first time, that the binding of activator protein-1 (AP-1)-DNA in rat uterus and the estrogen-sensitive areas of the hypothalamus, as measured by electrophoretic mobility shift assay, is increased 2 h after intraperitoneal injection of [D-Met^2,Pro^5]enkephalinamide. The effect was prevented by the opiate antagonist naltrexone given 30 min before the administration of [D-Met^2,Pro^5]enkephalinamide, suggesting the involvement of opioid peptide receptors in the observed effects. The present findings support the role of opioid peptides in the regulation of transcription in estrogen-sensitive cells.

Keywords: Opioid peptide; AP-1 (activator protein-1); Electrophoretic mobility shift assay; Transcriptional regulation; Uterus, rat; Hypothalamus, rat

1. Introduction

The regulation of reproduction is mediated in part through opioid peptides, which may operate at different levels. Opioid peptides tonically inhibit luteinizing hormone-releasing hormone (LHRH) secretion in the hypothalamus, and consequently, luteinizing hormone (LH) secretion in the pituitary (for review, see Dyer and Bicknel, 1989). The expression of the genes of endogenous opioid peptide precursors and opioid receptors in the uterus has been reported (Baraldi et al., 1985; Jin et al., 1988; Low et al., 1989).

The physiological roles of endogenous opioid peptides in the uterus are still debated. There are some convincing data about their regulatory role in uterine contraction, in local pain relief during pregnancy and partition, and, according to our previous studies, in the control of cell proliferation. Estrogen-stimulated cell proliferation in rat uterus was inhibited by a single injection of [D-Met^2,Pro^5]enkephalinamide (Ördög et al., 1992; Vértes et al., 1993).

The molecular mechanism through which the endogenous opioid peptides act on cell proliferation in the uterus is unknown. According to our earlier studies, opioid peptide-estrogen antagonism can be observed at different levels in estrogen-sensitive cells, suggesting a possible interaction between estrogen and opioid peptides in the regulation of transcription as well.

Transcription in the uterus is regulated in several ways. The main regulators are the gonadal steroids, of which estradiol is the most important. Estradiol influences gene expression by binding to receptors, whose DNA-binding domains attach to the estrogen-responsive elements (ERE) of the target genes. According to recent findings, the transactivation domains of the estrogen receptors can also modify transcription through the increased expression of the immediate-early genes, the products of which are transcription factors, the Fos and Jun proteins. These transcription factors form homo- and/or heterodimers and then bind to specific activator protein-1-DNA binding sequences on the promoter region (Philips et al., 1998).

* Corresponding author. Tel.: +36-72-326-222 then 1554; fax: +36-72-315-714.
E-mail address: oszter@physiol.pote.hu (A. Oszter).

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The opioid peptides exert their function by interacting with cell-surface receptors, which have been classified in three groups, μ-, δ-, and κ-opioid receptors. They are G-protein-coupled receptors. Several data suggest that opioids regulate gene expression in neural cells; however, the precise mechanism is not clear. Several pathways can be considered, among others, the cAMP-dependent mechanism, AP-1-DNA binding, and the involvement of different post-translational modifications (Tencheva et al., 1997).

Most studies of the effect of opioid peptides and the mechanism of their action were done primarily with neural cells. There is less data available about the mechanism of action of endogenous opioid peptides in non-neural tissues. The aim of the present experiment was to investigate the effect of opioid peptide on AP-1-DNA binding activity in the rat uterus and to compare it with binding in the estrogen-sensitive areas of the hypothalamus.

2. Materials and methods

2.1. Chemicals

[D-Met^2,Pro^5]enkephalinamide, a highly active μ- and δ-opioid receptor-selective enkephalin analogue (Bajusz et al., 1977), was a generous gift from Dr. S. Bajusz (Institute for Drug Research, Budapest, Hungary). All other chemicals, unless stated otherwise, were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals and drug treatments

Two-month-old CFY female rats were used. The animals were ovariectomized under light ether anaesthesia 10 days before the experiments. The rats were housed in temperature-controlled animal quarters under a 12-h light–dark cycle and maintained on ad libitum food and water. The animals were treated with [D-Met^2,Pro^5]enkephalinamide intraperitoneally at a dose of 50–200 μg/100 g BW at 1, 2, and 24 h before killing. To examine the antagonistic effect of naltrexone in some experiments, the drug was injected 30 min before [D Met^2,Pro^5]enkephalinamide. The controls received 0.15 M NaCl solution. The animals were killed by decapitation. The brains and cerebellums were quickly removed, cleared of adhering meninx, and washed thoroughly with ice cold physiological saline solution to remove contaminating blood. The hypothalamus was cut out as a block, and the estrogen sensitive areas were dissected by a crude dissection technique as described previously (Vértes et al., 1995). Tissue fragments containing estrogen sensitive hypothalamic regions (preoptic area, anterior hypothalamus, mediobasal hypothalamus, median eminence) were used for the analysis. The uteri and the small intestines were removed, cleared of adhering fat, and weighed after extrusion of intraluminal fluid and blotting. All procedures were carried out at 0–4°C unless stated otherwise.

2.3. Preparation of nuclear protein extracts and electrophoretic mobility shift assay

The preparation of nuclear protein extracts from fresh tissue fragments was carried out as previously described (Zhu and Pfaff, 1998). Briefly, dissected tissues were homogenized in a Polytron homogenizer with 2 vol of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM KCl, 0.5 mM phenylmethylsulfonyl-fluoride, 1 μg/ml of pepstatin A, 10 μg/ml of leupeptin and 0.1 mM *p*-aminobenzidine). After centrifugation for 20 min at 4°C, the supernatants were removed and the nuclear pellets were further extracted in 1.5 vol of buffer B (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl-fluoride, 1 μg/ml pepstatin A, 10 μg/ml of leupeptin and 0.1 mM *p*-aminobenzidine and 25% glycerol). The nuclear extracts were aliquoted, frozen and stored at −70°C until analysis. The protein concentration was quantitated by the BioRad protein assay.

The DNA binding reaction was performed in a total volume of 30 μl containing 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 4% glycerol, 5 mM dithiothreitol, 2 μg poly (dI-dC), 10–20 μg of nuclear protein extracts and 0.2 ng of ^{32}P-labelled oligonucleotide probe, and incubated at room temperature for 30 min. The DNA-protein complexes were separated on 5% non-denaturating polyacrylamide gels by electrophoresis. Gels were dried and quantitated by a Cyclone (BioRad) phosphor imager. A double-stranded AP-1 oligonucleotide probe (5′-GCAATTATGAGTCA-GTTTGC-3′) containing the AP-1 site of the transin promoter and 5′-CAGACAGCGTGGGCTGTGGC-3′ containing the Zif268 binding site for competition studies were used in the experiments (Promega, Madison, WI, USA).

2.4. Statistics

The data are presented as means ± S.E. of at least three experiments. Group differences were analyzed by analysis of variance (ANOVA) followed by Student–Newman–Keul's multiple range test. Differences were considered to be statistically significant at the $P < 0.05$ level.

3. Results

The specificity of AP-1-DNA binding activity was evaluated by competition assay using the uterine and hypothalamic nuclear extract. As shown in Fig. 1, the addition of a specific cold AP-1 oligonucleotide, but not a non-specific

oligonucleotide, to the DNA binding reaction resulted in inhibition of AP-1 binding with minimal changes in non-specific binding.

The AP-1-DNA binding activity was detected in the nuclear fraction of both tissues. In agreement with the results of others (Zhu and Pfaff, 1998), the basal level of AP-1 binding in the uterus of ovariectomized rats was significantly higher than that in the hypothalamus (uterus:hypothalamus ratio was 3.53 ± 0.26) (Fig. 1). The injection of [D-Met^2,Pro^5]enkephalinamide resulted in an approximately 50% (52 ± 8) increase in AP-1-DNA binding in the uterus. The injection of potent opioid peptide antagonist naltrexone 30 min before the administration of [D-Met^2,Pro^5]enkephalinamide abolished the increase in AP-1-DNA binding; naltrexone alone failed to affect binding (Fig. 1A).

The changes found in the estrogen-sensitive areas of the hypothalamus were similar to those of the uterus. AP-1-DNA binding activity was also increased after [D-Met^2,Pro^5]enkephalinamide administration, but the rate of the change was higher: AP-1-DNA binding activity increased by approximately 75% (70 ± 22) compared with the control values. Administration of naltrexone before [D-Met^2,Pro^5]enkephalinamide prevented the increase in AP-1-DNA binding activity. Naltrexone administration alone in the hypothalamus was also ineffective (Fig. 1B). AP-1-DNA binding activity in nuclear protein extracts from intestine and cerebellum were used for controls. Changes in binding activity after [D-Met^2,Pro^5]enkephalinamide administration were detected only in the cerebellum (Fig. 2).

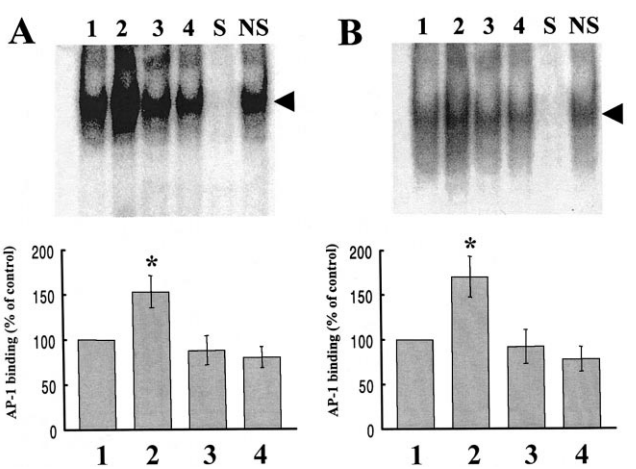

Fig. 1. Effect of [D-Met^2,Pro^5]enkephalinamide on the AP-1 binding activity of rat uterine (A) and hypothalamic (B) nuclear extracts. The AP-1-DNA binding activity was analyzed by electrophoretic mobility shift assay. *Lane 1*: ovariectomized vehicle control animals; *Lane 2*: ovariectomized rats treated with [D-Met^2,Pro^5]enkephalinamide (100 μg/100 g BW i.p.) 2 h before killing; *Lane 3*: ovariectomized rats treated with naltrexone 30 min before [D-Met^2,Pro^5]enkephalinamide administration; *Lane 4*: naltrexone (100 μg/100 g BW, i.p.) 2 h before killing. S: specific control; NS: non-specific control. The arrowheads indicate the specific AP-1-DNA complexes. The data are presented as percentages of ovariectomized vehicle control (100%). The values are the means ± S.E. of three experiments, $^* p < 0.05$.

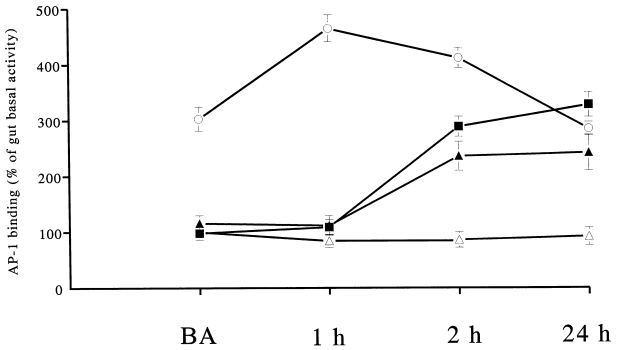

Fig. 2. Time-dependent effect of [D-Met^2,Pro^5]enkephalinamide on AP-1-DNA binding activity in nuclear protein extracts of ovariectomized rat tissues. The animals were treated with [D-Met^2,Pro^5]enkephalinamide (100 μg/100 g BW, i.p.) at the indicated time before killing. BA = basal activity of AP-1 binding in the examined tissues. Open triangle = gut, open circle = uterus, filled square = cerebellum, filled triangle = hypothalamus. The data are presented as percentages of gut basal activity (100%). The values are the means ± S.E. of three experiments.

The time course of the effect of [D-Met^2,Pro^5]enkephalinamide administration on AP-1-DNA binding is shown in Fig. 2. The maximal response was found at 2 h after [D-Met^2,Pro^5]enkephalinamide treatment in the hypothalamus and in the uterus followed by a decrease up to 24 h; in the cerebellum, the highest values in our experiments were found at 24 h. No changes were found in the intestinal tissues.

[D-Met^2,Pro^5]enkephalinamide, at a dose of 50 μg/100 g BW, stimulated AP-1 binding in all responsive tissues 2 h after its administration. The effect was maximal in the uterus. Increasing the amount of [D-Met^2,Pro^5]enkephalinamide did not change the evoked responses in the uterus, but in the hypothalamus and cerebellum a dose of 100 μg/100 g BW resulted in an approximately 25% and 37% increase, respectively. The administration of 200 μg/100 g BW [D-Met^2,Pro^5]enkephalinamide failed to induce further changes (data not shown).

4. Discussion

As shown by our results, AP-1-DNA binding affinity in the rat uterus and the estrogen-sensitive areas of the hypothalamus was increased by a single injection of [D-Met^2,Pro^5]enkephalinamide. These effects were prevented by the administration of the opiate antagonist naltrexone given 30 min before [D-Met^2,Pro^5]enkephalinamide, suggesting the involvement of opioid peptide receptors in the observed effects.

The AP-1 transcription factors are groups of proteins that recognize and bind to specific AP-1-DNA sequences of genes. The family consists of two groups of proteins,

the Fos- and Jun-related proteins. These proteins are usually expressed at low basal levels and can be induced by several factors in different cells. An increase in the amount of AP-1 transcription factors is usually followed by an increase in DNA binding activity.

The effect of opioid peptides on the control of transcription has been extensively studied. Opiates stimulate the brain region-specific expression of the *fos* and *jun* genes (Liu et al., 1994; Couceyro and Douglas, 1995) and AP-1 binding activity, which suggests that they play a role in the development of tolerance and dependence by causing opioid receptor down and up regulation (Tencheva et al., 1997) and in the induction of certain behavioral alterations (Liu et al., 1994).

In the estrogen-sensitive areas of the hypothalamus, similar to the cerebellum, [D-Met^2,Pro^5]enkephalinamide stimulated AP-1-DNA binding activity; however, the time course and dose–response curves were different. [D-Met^2,Pro^5]enkephalinamide did not influence AP-1 binding in the opioid peptide-rich gut tissues, but in the uterus the effect was similar to that in the hypothalamus. According to our former findings, endogenous opioid peptides inhibit estradiol-induced cell proliferation in the uterus.

The cellular proliferation and differentiation processes are regulated by different hormonal factors, which bind to their specific receptors and initiate intracellular cascades and thus initiate the changes in the activity of the transcription factors in the nuclear compartments (Bamberger et al., 1996). The AP-1 proteins are prototypes of the mitogen-activated transactivators and they influence cell proliferation in many cells. In the uterus and hypothalamus AP-1-DNA binding activity was also dependent on estrogen. The effect of estrogen on the induction of AP-1 proteins and DNA binding activity was different in the hypothalamus and in the uterus. On estradiol administration in the hypothalamic estrogen-sensitive areas, AP-1 binding decreased while in the uterus it increased (Zhu and Pfaff, 1998). How the increased AP-1-DNA binding found in the present experiment is related to the effects of estrogen is not known. It is also a question which genes were influenced by endogenous opioid peptides and estrogen in the tissues examined.

The novel finding in this study is that [D-Met^2,Pro^5]enkephalinamide in the uterus increased AP-1-DNA binding activity, similar to that in the hypothalamus. On the basis of these findings, it could be supposed that opioid peptides act on transcription in certain non-neural cells as well. The effects in the studied tissues were abolished by naltrexone, which suggests that opioid receptors and consequent post-receptor signaling mechanisms contribute to the regulation of transcription.

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