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Opioid peptides inhibit the action of oestradiol on human myometrial cells in culture*

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The effect of opioid peptides on cultured, oestradiol-stimulated human myometrial cells was examined. Oestradiol increased cell densities in mixed-cell (smooth muscle cells + stromal fibroblasts) cultures by 40%. This oestradiol-induced stimulation of cell proliferation was decreased to control values by D-met[2]-pro[5]-enkephalinamide. The half-effective inhibitory concentration of enkephalinamide was 0.3 nmol/l. The opioid-induced inhibition of cell proliferation was blocked completely by the specific opiate receptor antagonist naloxone, while naloxone did not have any effect on its own. This opioid effect was mediated dominantly by the mu opiate receptor. The optimal concentration for oestradiol to stimulate uterine cell proliferation was 2.2 nM. The basal rate of cell proliferation was not affected by enkephalinamide. In saturation experiments, the parameters of specific [^{3}H]-naloxone binding were: dissociation constant = 1.02 nM, maximal binding capacity = 2910 binding sites/cell, Hill coefficient = 1.029. In human myometrial pure smooth muscle cell cultures, oestradiol decreased the proliferation of cells. Progesterone potentiated these oestradiol effects, but had no effect on its own. Enkephalinamide was also able to block the effects of oestradiol, but naloxone did not antagonize it. In summary, here we present a novel inhibitory role of endogenous opioid peptides in the regulation of cell growth and proliferation in the human uterus.

Key words: human/oestradiol/opiates/proliferation/uterus

Introduction

Endogenous opioid peptides (Wahlstrom *et al*., 1985; Li *et al*., 1991), as well as functional opioid receptors (Baraldi *et al*., 1985; Vertes *et al*., 1986, 1993), mainly of the μ and, to a lesser extent, of the κ receptor subtypes (Wittert *et al*., 1996) and their mRNA (Douglass *et al*., 1987; Jin *et al*., 1988; Muffly *et al*., 1988; Low *et al*., 1989; Wittert *et al*., 1996; Zhu and Pintar, 1998) are present in the uterus and are regulated by ovarian steroids (Baraldi *et al*., 1985; Wahlstrom *et al*., 1985; Vertes *et al*., 1986; Jin *et al*., 1988; Low *et al*., 1989).

The major physiological roles of endogenous opioid peptides in the uterus are: the inhibition of uterine contractions (Ohia and Laniyonu, 1989; Poli *et al*., 1990; Faletti *et al*., 1992), local pain relief during pregnancy and parturition (Kimball *et al*., 1984; Baraldi *et al*., 1985) and the inhibition of uterine cell proliferation (Ordog *et al*., 1992; Kornyei *et al*., 1997).

Previously, we have described the inhibition of cell proliferation by endogenous opioid peptides both *in vivo* and *in vitro* in the adult (Ordog *et al*., 1992, 1993; Kornyei *et al*., 1997) and developing (Vertes *et al*., 1995b, 1996) rat uterus. However, no data are available on the role of endogenous opioid peptides in the regulation of cell proliferation in the human uterus. In our present experiments, we examined the effect of opioid peptides on the oestradiol-stimulated cell proliferation of human myometrial cells in culture. Since oestradiol-induced uterine cell proliferation is known to be mediated by stromal cells (Schatz *et al*., 1984; Cooke *et al*., 1997), we also analysed the effect of oestradiol and endogenous opioid peptides in mixed-cell and pure smooth muscle cell cultures of human myometrium.

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Materials and methods

Tissues

Human uterine tissue collection, cell dispersion and culture was essentially the same as described previously (Kornyei *et al*., 1993). Briefly, myometrial specimens were obtained from pre-menopausal women undergoing hysterectomy for benign indications with no history of hormonal treatment. The use of the tissues was approved by our institutional Human Studies Committee.

Dispersion of cells

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. To obtain pure smooth muscle cell cultures, the myometrial cells were dispersed by 18 h digestion with 2 mg type XI collagenase/ml and 0.01 mg type I DNase/ml (Kornyei *et al*., 1993). The digests were filtered through cell dissociation sieves. The filtrates were centrifuged for 5 min at 300 *g* and the cell pellets were washed three times with Hank's medium.

To obtain mixed cell cultures (smooth muscle cells with stromal

fibroblasts), we used two 50 ml sterile centrifuge tubes containing 1.5–2.5 g of the 1–2 mm^3 tissue blocks each. The following limited enzymatic digestions were performed: 15 min at 4°C in 10 ml Hank's balanced salt solution without calcium and magnesium ions (HBSS-0) containing 0.1 mg trypsin–EDTA/ml, 5 μg type I DNase/ml, 2% HEPES at pH 7.4 with occasional intensive vortexing. The digests were filtered through cell-dissociation sieves and tissue pieces were placed back into the tubes with fresh enzyme solution. The second and third incubations were of the same kind, the 4th and 5th were performed at 20°C in the same solution. The remaining tissue blocks were then placed into regular Hank's balanced salt solution (HBSS-2) containing 2 mg type XI collagenase/ml, 0.01 mg type I DNase/ml, 2% HEPES at pH 7.4, and were incubated at 37°C for 1 h. The following final incubation was the same, where practically no tissue blocks remained. The resulting pellets were washed three times, then combined and plated onto 150 cm^2 Corning cell culture flasks.

Culturing the cells

The cells were cultured in Waymouth's medium (WM) containing 10% fetal bovine serum (FBS), 2% antibiotic–antimycotic solution, 1% sodium pyruvate, 2% minimum essential medium (MEM) amino acids, 1% non-essential amino acids, 1% MEM vitamins, 0.2 mg kanamycin/ml and 2% HEPES at pH 7.4 in an atmosphere of 5% CO_2 at 37°C.

Checking cell type content of cell lines

The purity of human myometrial smooth muscle cells and the cell type content of the mixed cultures were determined by immunocytochemistry using the anti-α smooth muscle actin antibody for smooth muscle cells, anti-type I collagen for fibroblasts, anti-CD68 antibody for macrophages and anti-CD34 antibody for endothelial cells. The immunohistochemical reactions were detected by the avidin–biotin–peroxidase (ABC) method (Kornyei *et al*., 1993).

Experimental treatments

For both the mixed-cell and smooth muscle cell experiments, 2000 dispersed viable cells per cm^2 were plated onto 25 cm^2 Corning cell culture flasks. All the experiments presented in this paper were performed during the first (mixed) or the first to third (smooth muscle) subculture of the cells. Hormones (oestradiol and progesterone) and opioid peptide treatments, D-Met^2, Pro^5-enkephalinamide (ENK), D-Ala^2, *N*-Me-Phe^4-Gly^5-ol-enkephalin (DAMGO), D-$Pen^{2,5}$-enkephalin (DPDPE), porcine dynorphin-A (DYN-A), and opiate receptor antagonist naloxone (NAL), were added to the culture medium in the flasks on culture day 2 when cell attachment was complete. The culture medium (see above) was changed every 48 h and the treatments were present during the entire duration of culture. As fetal bovine serum might contain oestrogenic hormones and thus might bias our observed proliferative effects, we used charcoal-stripped FBS in some parallel early experiments with no change in the final results.

Counting the cells

The cultures were stopped just before confluency was reached on about day 9 to avoid bias from contact inhibition. At the end of the experiments, culture medium was replaced with basic WM and the flasks were kept at 4°C until all treatment groups could be counted one at a time. Detachment of the monolayers was performed by trypsinization. The cells were counted in haemocytometers by two independent investigators not knowing the others' results, or by a Coulter counter ZM (Coulter Electronic, Hia Leah, FL, USA) equipped with a 100 μm diameter aperture tube.

Measuring DNA content

To further control the accuracy of our measuring techniques, in some experiments the DNA content of one flask from the groups were also determined by Burton's method (Burton, 1956).

Detecting cell size

The cell size was determined manually on photographs or by using the Coulter counter set to size cells at ⩾8 μm.

Karyotype determination

Chromosome analysis was performed by examining ploidy of the cells. Protein concentrations were measured by the Lowry method (Lowry *et al*., 1951).

Radioligand binding assay

For [^{3}H]-naloxone saturation analyses a sufficient amount of cells were raised in 150 cm^2 (n = 10) flasks for the end of 2nd subculture phase. The confluent monolayers were removed by cell scrapers. Homogenization was performed in TMN buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl at pH 7.4) with an Ultra-Turrax homogenizer (Janke and Kunkel, IKA-WERK, Staufen, Germany) on low speed and constant cooling. Following centrifugation the 800 g supernatant was taken as the cytoplasmic fraction containing the cell membrane fragments. The radioligand binding assay was as described previously (Vertes *et al*., 1993). Briefly, samples (0.5 ml) in triplicate, were incubated with 0.5–5 nM [^{3}H]-naloxone (MTA-SzBK, Szeged, Hungary) with or without 1000-fold excess of unlabelled naloxone (Endo Laboratories, Garden City, NY, USA) at 25°C for 1 h. At the end of incubation, samples were rapidly filtered through Whatmann GF/B glass microfibre filters and washed three times with 5 ml ice-cold buffer. Filters were placed into counting vials, air-dried overnight, then radioactivity was measured in a Packard Tri-Carb model 2100TR liquid scintillation spectrometer (Canberra-Packard Kft., Budapest, Hungary).

Statistical analyses

All the experiments were performed in triplicate and repeated at least three times on cells from different tissue specimens. All the experiments could not be performed on the same batches of cells. Each procedure was performed at 4°C unless otherwise stated. The results (expressed as 10^3 cells/cm^2 units) are the mean ± SE of six data points from one representative experiment. A two tailed t-test, or analysis of variance followed by a post-hoc Student–Newman–Keul's multiple range test (SNK-MRT) (Dowdy and Wearden, 1983) were used for data analysis. Experimental data from [^{3}H]-naloxone saturation analyses were analysed by our REC.ANAL computer program package (Kornyei, 1987).

Results

Human myometrial cell lines were successfully established and maintained in culture. The results of immunocytochemical reactions performed on sections of pellets from smooth muscle cell cultures showed that >99% of the cells were immunostained with the antibody specific for smooth muscle cells but not with those specific for fibroblasts, macrophages or endothelial cells. Mixed-cell type cultures contained roughly equal amounts of smooth muscle and stromal cells (data not shown).

Monolayers of mixed-type cultured cells grew to confluency in 10–12 days with an average population doubling time of

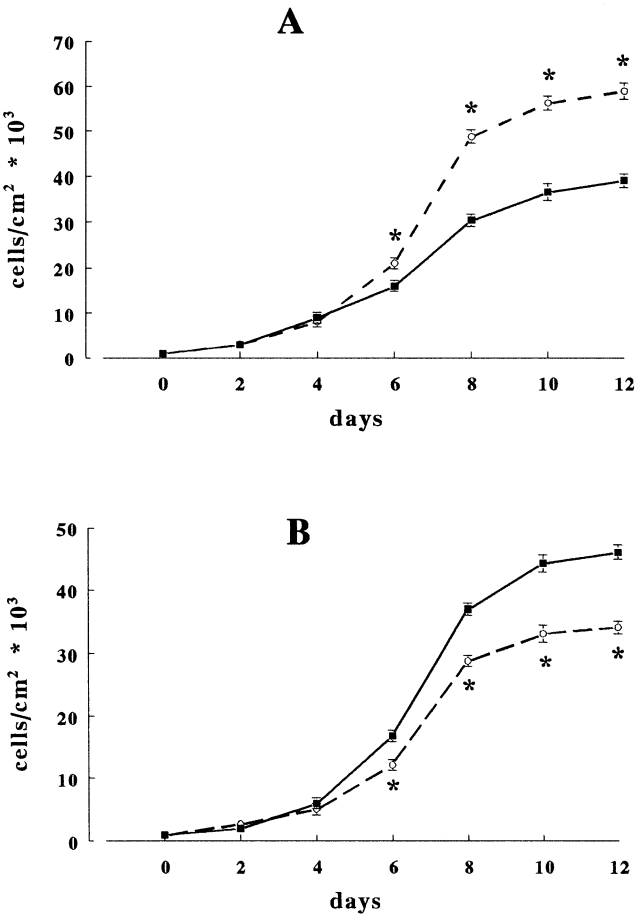

Figure 1. Time-dependent inhibitory effect of D-met^2-pro^5-enkephalinamide (ENK) on (**A**) the oestradiol-induced cell proliferation in human myometrial mixed-cell cultures and (**B**) on the oestradiol-triggered decrease of cell proliferation in pure human myometrial smooth muscle cell cultures. --○-- = oestradiol 2.2 nM alone; –■– = oestradiol 2.2 nM plus ENK (30 nM). The oestradiol + ENK data points presented here overlapped the control, treatment points and curves not shown; $n = 6$ each, in mixed-cell cultures. (**A**) analysis of variance, $F = 0.02$, 0.39, 13.48, 79.57, 58.18 and 44.61, in pure human myometrial smooth muscle cell cultures; (**B**) analysis of variance, $F = 0.88$, 0.43, 27.11, 58.32, 26.41 and 44.27 for the 2, 4, 6, 8, 10 and 12 day data sets respectively. *Value significantly different from control ($P < 0.01$); post-hoc Student–Newman–Keul's multiple range tests.

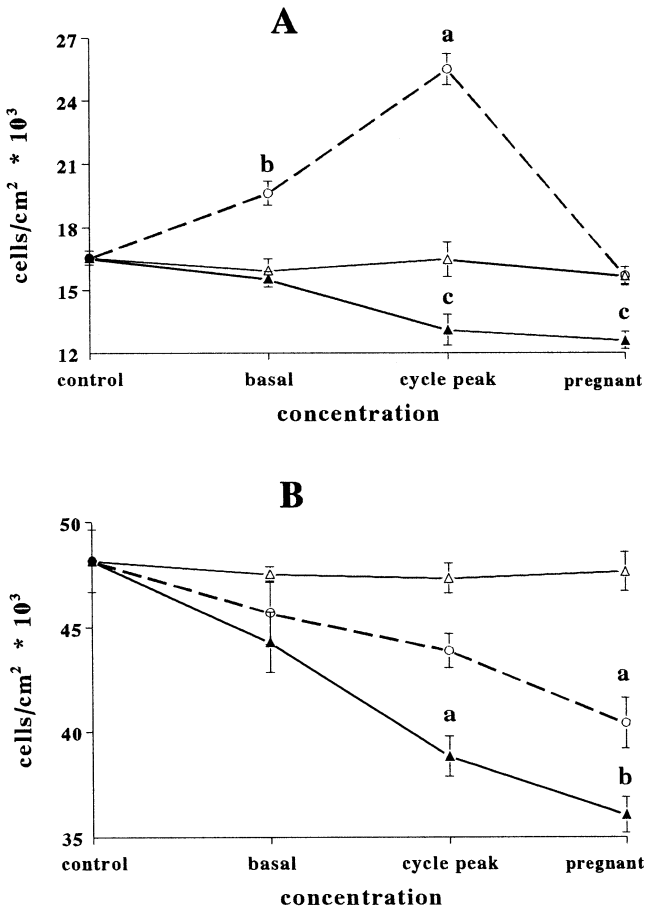

Figure 2. Concentration-dependent effect of sex steroid hormones on (**A**) cell proliferation in human myometrial mixed-cell cultures and (**B**) pure human myometrial smooth muscle cell cultures. ● = control, no treatment; ○ = oestradiol, △ = progesterone; ▲ = oestradiol + progesterone together. Culture medium contained the concentrations of hormones: basal, 0.2 nM oestradiol and/or 1.6 nM progesterone; cycle peak, 2.2 nM oestradiol and/or 40 nM progesterone; and pregnant, 220 nM oestradiol and/or 800 nM progesterone; $n = 6$ each. (**A**) analysis of variance, $F = 40.82$; (**B**) analysis of variance $F = 15.07$. Post-hoc Student–Newman–Keul's multiple range test: a, b, c are significantly different from control and from each other ($P < 0.01$).

1.5–2 days (Figure 1A). Oestradiol increased cell densities of mixed-cell cultures by 40% (Figure 1A). This oestradiol-induced stimulation of cell proliferation was decreased to control values by ENK (Figure 1A). This inhibition appeared after a 2–3 day latency period. The proliferation of pure smooth muscle cell cultures was decreased by oestradiol (Figure 1B). This oestradiol-induced slow-down of cell proliferation could be completely blocked by ENK (Figure 1B). Average population doubling time was 1.5–2.8 days, similar to the mixed-cell cultures above. It took 2–3 days for oestradiol and ENK to show detectable, statistically significant effects.

The most effective concentration for oestradiol to stimulate uterine cell proliferation in mixed-cell cultures was 2.2 nM (Figure 2A) while high concentrations failed to act. Progesterone did not alter cell densities, while the combination of oestradiol and progesterone decreased cell numbers at higher physiological concentrations (Figure 2A). In pure smooth muscle cell cultures (Figure 2B), oestradiol did not have a stimulatory effect on cell proliferation in basal and cycle peak concentrations, but rather decreased uterine cell proliferation at high physiological concentrations (Figure 2B). Progesterone alone did not alter cell densities, but when combined with oestradiol in cycle peak concentrations and especially in the high concentrations typical of pregnancy, together they significantly decreased cell numbers in the subconfluent monolayers (Figure 2B).

To check the possible background of this unusual sex steroid effect, we determined the cell size distributions of the smooth muscle cell cultures (Figure 3). The sex steroid hormone treatment resulted in the appearance of a population of giant-size cells (Figure 3B). Even the average cell size differences were apparent when expressed in pg protein/cell units (482 ±

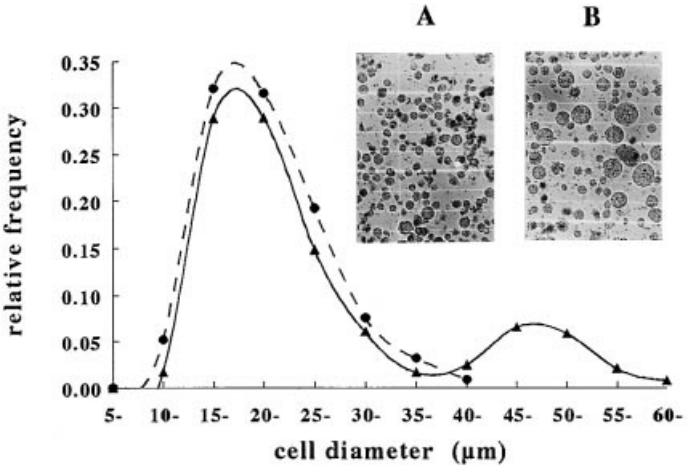

Figure 3. The effect of sex steroid hormones on the cell size distribution of pure human myometrial smooth muscle cell cultures. Diameters of cells were measured on photographs taken in haemocytometers containing the trypsinized cells (insert). --●-- = control, no treatment (mean 22.04 ± 0.41 µm, n = 392), see insert A. –▲– = 220 nM oestradiol and 800 nM progesterone pregnant concentrations were present in the medium (mean 25.79 ± 0.70 µm, n = 242), see insert B. Two tailed t-test: t = 4.81, significantly different ($P < 0.01$).

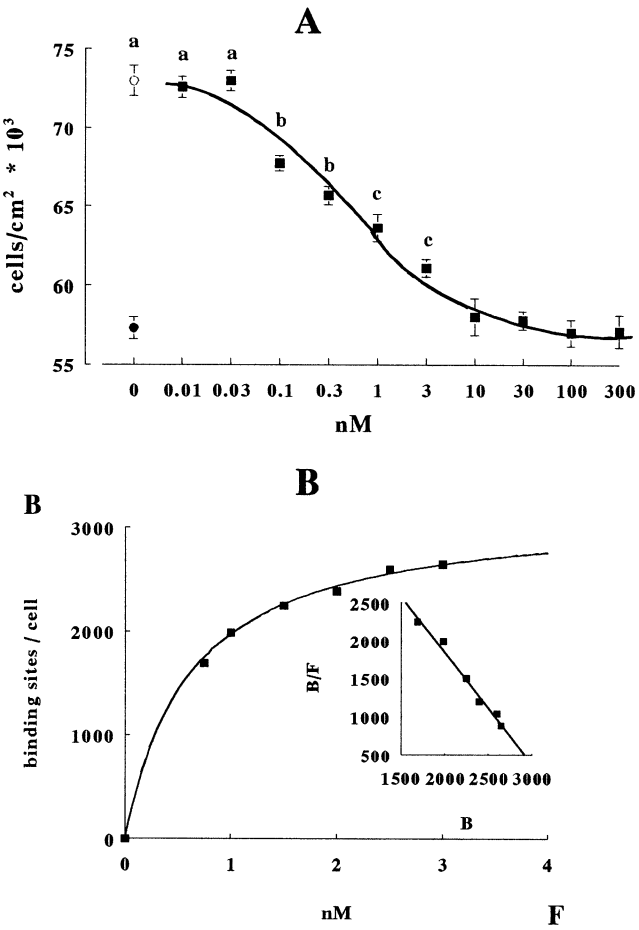

Figure 4. (**A**) Concentration dependent inhibitory effect of enkephalinamide (ENK) on the oestradiol-induced cell proliferation in human myometrial mixed-cell cultures. ■ = oestradiol 2.2 nM and enkephalinamide 0.01–300 nM; ○ = oestradiol 2.2 nM alone; ● = control, no treatment; n = 6 each. Analysis of variance, F = 67.53; post-hoc Student–Newman–Keul's multiple range tests, a, b, c are significantly different from control and from each other ($P < 0.01$). (**B**) Specific [^{3}H]-naloxon binding in cytoplasmic fractions of human myometrial mixed-cell culture monolayers. Binding parameters: K_D= 1.02 nM, B_{max}= 2910 binding sites/cell, Hill coefficient = 1.029. Insert: Scatchard plot.

19 versus 579 ± 24, n = 6 each; t = 3.169, significantly different, $P < 0.01$). The karyotype of both the normal (n = 10) and giant (n = 10) cells were found to be regular diploid human XX chromosome sets.

The half-effective inhibitory concentration of ENK to inhibit the oestradiol-induced cell proliferation in mixed-cell cultures was 0.3 nM (Figure 4A). Concentrations of 10 nM and above completely blocked the effect of oestradiol. The presence of functional opiate receptors was also studied in mixed-cell cultures. In saturation experiments (Figure 4B), the parameters of specific [^{3}H]-naloxone binding were: K_D = 1.02 nM, B_{max} = 2910 binding sites/cell, Hill coefficient = 1.029. Dissociation constant data obtained here are in good agreement with the IC_{50} values (Figure 4A).

The opioid-driven inhibition of the oestradiol-induced cell proliferation in mixed-cell cultures (Figure 5, black columns) was completely blocked by the specific opioid receptor antagonist naloxone (NAL), while naloxone did not have any effect on its own either on oestradiol stimulated, or on basal growth rate. The basal proliferation of the cells was not affected by the presence of ENK. Occasionally-performed parallel DNA determinations showed the same differences according to the several treatments in the range of 40–200 µg DNA/flask and, hence, 7–8 pg DNA per cell values could be obtained for each group. In pure smooth muscle cell cultures the blocking effect of ENK could not be antagonized by NAL (Figure 5, cross-hatched columns), thus the ENK effect seems not to be mediated by the classical cell membrane opiate receptors in human myometrial smooth muscle cells. The basal proliferation rate was not affected by the presence of ENK and/or NAL. Similar to data above, no interference was visible between oestradiol-mediated negative proliferative effect seen here and NAL.

The involvement of the opiate receptor subtypes in the action of ENK was also dependent on the type of cell cultures used (Figure 6). Similar to data presented above, ENK completely inhibited the oestradiol-induced cell proliferation stimulation in mixed-cell cultures (Figure 6, black columns), and successfully antagonized the oestradiol-driven decrease of proliferation in pure smooth muscle cell cultures (Figure 6). Selective opioid peptides were added to the culture medium: the µ-specific DAMGO was able to block the oestradiol-stimulated cell proliferation in mixed-cell cultures (Figure 6, black columns), while it failed to act in pure smooth muscle cell cultures (Figure 6, cross-hatched columns). DPDPE did not change cell densities showing the lack of involvement of δ-opiate receptors. Though not significant ($P > 0.01$), the κ-specific dynorphin-A showed an over-stimulatory effect on the oestradiol-induced cell proliferation of mixed-cell cultures. In addition, this slight stimulatory effect of dynorphin-A was always visible in the oestradiol-treated smooth muscle cell cultures (Figure 6).

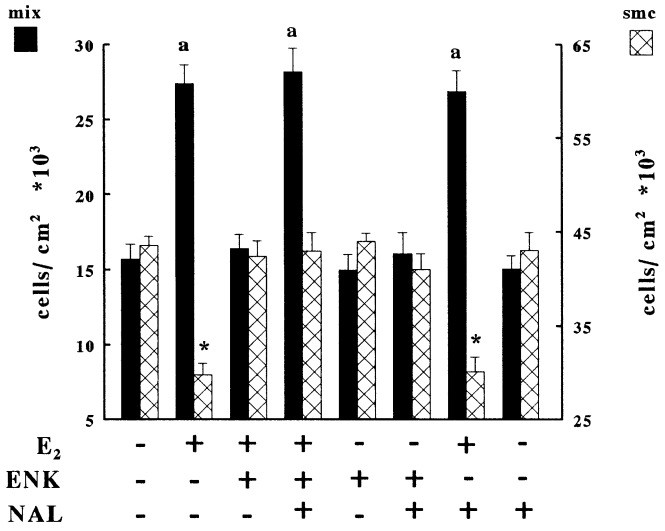

Figure 5. Blockage of the inhibitory effect of enkephalinamide (ENK) on the oestradiol-stimulated cell proliferation in human myometrial mixed-cell cultures (black columns from left axis) and the lack of blockage of ENK's inhibitory effect on the estradiol-driven decrease of cell proliferation speed in pure human myometrial smooth muscle cell-cultures (cross-lined columns from right axis) by the opiate receptor antagonist naloxone (NAL). The following concentrations were present in the culture medium: oestradiol, 2.2 nM; NAL, 30 nM; ENK, 30 nM. Black columns ($n = 6$): analysis of variance, $F = 26.63$; post-hoc Student–Newman–Keul's multiple range test, letters 'a' mark values significantly different from corresponding control ($P < 0.01$). Cross-lined columns ($n = 6$), analysis of variance, $F = 12.39$, post-hoc Student–Newman–Keul's multiple range test, *Value significantly different from corresponding control ($P < 0.01$).

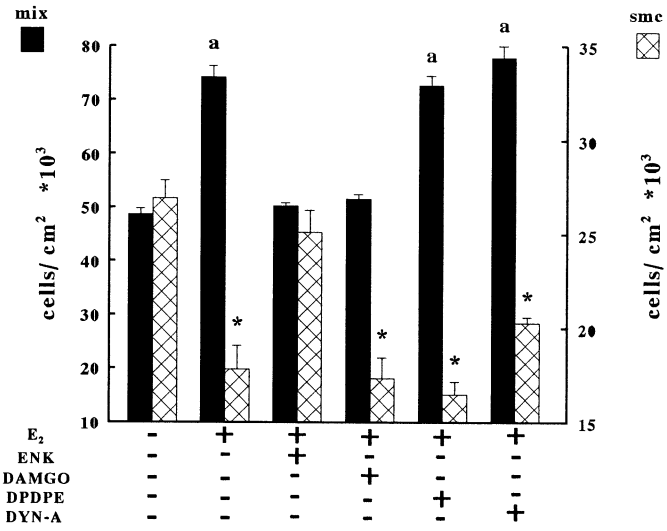

Figure 6. Opiate receptor subtypes involved in the inhibitory effect of enkephalinamide (ENK) on the actions of oestradiol in human myometrial cell cultures, in the presence (+) or absence (–) of oestradiol (E_2) 2.2 nM; ENK 100 nM; D-Ala^2, *N*-Me-Phe^4-Gly^5-ol-enkephalin (DAMGO) 100 nM (μ selective); D-Pen^{2,5}-enkephalin (DPDPE) 100 nM (δ selective) and dynorphin-A (DYN-A) 100 nM (κ selective). Black columns from left axis mark mixed cell cultures, analysis of variance, $F = 78.95$; post-hoc Student–Newman–Keul's multiple range test, values marked by letters 'a' are significantly different from control ($P < 0.01$). Cross-lined columns from the right axis are pure smooth muscle cell cultures, analysis of variance, $F = 18.6$, post-hoc Student–Newman–Keul's multiple range test; *value is significantly different from control ($P < 0.01$).

Discussion

An increasing number of data show that the endogenous opioid peptides have an inhibitory effect on cell proliferation, both within the nervous system (Vertes *et al*., 1982; Zagon and McLaughlin, 1991) and in some non-neural tissues, including rat uterus (Ordog *et al*., 1992, 1993; Vertes *et al*., 1995b, 1996; Kornyei *et al*., 1997). Endogenous opioid peptides (Wahlstrom *et al*., 1985; Li *et al*., 1991), functional opioid receptors (Baraldi *et al*., 1985; Vertes *et al*., 1986, 1993) and their mRNA messages (Douglass *et al*., 1987; Jin *et al*., 1988; Muffly *et al*., 1988; Low *et al*., 1989; Wittert *et al*., 1996; Zhu and Pintar, 1998) are all present in the uterus and their concentrations are regulated by ovarian steroids (Baraldi *et al*., 1985; Wahlstrom *et al*., 1985, Vertes *et al*., 1986; Jin *et al*., 1988; Low *et al*., 1989). The inhibitory role of endogenous opioid peptides in the regulation of cell proliferation has also been described in established human breast cancer cell lines (Maneckjee *et al*., 1990; Hatzoglou *et al*., 1996). We were led to the present experiments by the lack of data on the role of opioid peptides in the regulation of cell proliferation in the human uterus.

Our present study has described the novel role of endogenous opioid peptides in the regulation of cell proliferation in the normal human uterus. We have demonstrated that endogenous opioid peptides inhibit the oestradiol-induced cell proliferation of human myometrial mixed-cell cultures in a time- and concentration-dependent manner, suggesting a direct mechanism of action at the cellular level. The effective concentrations were within normal physiological ranges and naloxone could completely eliminate the inhibitory effect, suggesting a receptor-mediated mechanism of action. Our results, together with previously published data, are consistent with the fact that the oestradiol-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; Ordog *et al*., 1993; Hatzoglou *et al*., 1996; Vertes *et al*., 1996; Kornyei *et al*., 1997).

In our oestradiol dose-dependency experiments on human myometrial mixed-cell cultures, oestradiol-stimulated cell proliferation in the physiological nanomolar range characteristic of the menstrual cycle. The observed decrease in the stimulatory ability of oestradiol at high, pregnancy-type concentrations may also be consistent with physiological regulation. In agreement with data from the literature (Nardulli and Katzenellenbogen, 1986; Borras *et al*., 1994), we conclude that the long-term, high-dose oestradiol treatments present in the medium might cause some type of down-regulation of one or more parts in the mechanism of action of oestradiol. These high, pregnancy-type concentrations of oestradiol even failed to act on nitric oxide production of cultured pregnant rat myometrial cells (Gangula *et al*., 1997). Progesterone alone did not change cell concentrations, but in combination with oestradiol, decreased the speed of cell proliferation. This observation is in agreement with data from the literature, as progesterone has anti-oestrogenic effect on uterine cells and

functional progesterone receptors are synthesized in response to oestradiol (Savouret *et al*., 1994; Graham and Clarke, 1997; Kraus *et al*., 1997).

The mechanism of action of endogenous opioid peptides involves G_i proteins that decrease intracellular cAMP values, thus lowering the activity of cAMP-dependent kinases (Mansour *et al*., 1995; Satoh and Minami, 1995). As cAMP is known to activate the oestrogen receptors in the uterus (Aronica and Katzenellenbogen, 1993), the opioid-induced lack of sufficient cAMP supply might decrease the activity of the oestrogenic signalling system. On the other hand, the existence of membrane-bound oestrogen receptors has also been described; when activated by oestrogenic ligand they increase the cAMP values (Aronica *et al*., 1994; Revelli *et al*., 1998) and, by acting on the specific cAMP response elements, the transcription of specific genes is altered as well as the immediate ionic responses. Both mechanisms could explain a close cross-talk connection between the oestrogenic and opioid peptide signalling systems.

The opiate receptor subtypes involved in the inhibition of oestradiol-induced cell proliferation were found to belong mainly to the μ opiate receptor subtype. These findings are consistent with the presence of mainly μ, less δ and κ receptor mRNA messages in rat uterus (Wittert *et al*., 1996) and with the μ-preferring binding characteristics of ENK (Ronai *et al*., 1981). However, the present data differ from previous data obtained in rat uterus, with respect to the involvement of κ opiate receptor subtypes (Kornyei *et al*., 1997). Although not significant ($P > 0.01$), a slight proliferation over-stimulatory action of dynorphin-A in oestradiol-treated human uterine cultures could be observed, giving rise to the possibility that differences in signalling mechanisms may exist between rodent and human tissues; further experiments are required to clarify this.

The opioid peptides exert their action primarily by activating their cell membrane receptors that can be fully antagonized by NAL. In contrast to this, our present data showed that endogenous opioid peptides are able to inhibit the effects of oestradiol in pure smooth muscle cell cultures, but raising the possibility of a NAL-insensitive opioid action. On the other hand, the interaction of the oestradiol and endogenous opioid peptide systems can even be observed at the intracellular receptor level. In our previous binding studies, endogenous opioid peptides and oestradiol could displace each other from the type II oestradiol-binding sites, that are closely related to true uterine growth (Vertes *et al*., 1986; Garai *et al*., 1989). Based on our present results, we conclude that the additional intracellular localization of functional opiate receptors (Garai *et al*., 1989; Szucs *et al*., 1990; Belcheva *et al*., 1993; Vertes *et al*., 1995a) makes the receptoral intracellular cross-talk possible. We postulate that the binding spectra of ENK used in our experiments is wide enough to reach, and act on, these intracellular binding sites, in contrast to the selective specific peptides that bind only to their specific membrane receptor subtype.

Our present data, obtained in myometrial pure smooth muscle cell cultures, showed that cell densities of oestradiol-treated cultures were lower than those of the controls. Available data are consistent with the fact that oestradiol decreases the proliferation of target organ cells if no corresponding stromal cells are present (Fukamachi and McLachlen, 1991; Rossi *et al*., 1992; Kornyei *et al*., 1993). The well-known stimulatory effect of oestradiol on proliferation requires intact tissue structure or a mixture of the different types of cultured cells, to simulate the content of original tissues (Schatz *et al*., 1984; Astrahantseff and Morris, 1994, Cooke *et al*., 1997). In the myometrial mixed-cell cultures of the present study, the inhibition of smooth muscle cell proliferation and increase of cell size were not detectable. We suppose that these effects were possibly over-ridden by the paracrine mitogenic effects of stromal cells.

In addition to the down-regulatory effect of oestradiol, we have described the increase of size in a subset of smooth muscle cells as a result of the action of oestradiol in the human uterus. This effect was most pronounced when oestradiol was combined with progesterone at the high concentrations characteristic of pregnancy. This observation is in agreement with the fact that marked hypertrophy and less hyperplasia is known to occur in the myometrium of the pregnant uterus. Meanwhile, it is of interest that similar effects on cell size by human chorionc gonadotrophin have been described (Kornyei *et al*., 1993).

Differences in the way that oestradiol acts on different cell types and especially on their mixtures can be explained by possible alterations in the relative amount of the classical oestradiol receptor α and the novel β receptor forms (Mosselman *et al*., 1996; Enmark *et al*., 1997; Kuiper and Gustafsson, 1997; Ogawa *et al*., 1998). Since the amino acid sequence of human ER β with the ER α shows a high degree of conservation of the ligand-binding domain and of the DNA-binding domain (Couse *et al*., 1997; Pace *et al*., 1997; Pettersson *et al*., 1997; Tong *et al*., 1997), they have the same high affinity and specificity for oestrogenic ligands, bind to the same response elements initiating transcription of the same genes, but do not have conserved, variable regions in the A/B domain, the hinge region and the F-domain, they are markedly different targets for activation or inhibition by cross-talk mechanisms with other signalling systems.

In recent publications, negative regulators of transcription by oestradiol have also been demonstrated (McDonnell *et al*., 1992; Saatcioglu *et al*., 1994; Yen and Chin, 1994). In contrast to the positive regulatory elements with their stimulatory effects, these negative regulators might be responsible for oestradiol-induced direct inhibition of specific cellular functions. The availability of these negative regulatory sites might differ by cell types and might explain even the inhibitory actions of oestradiol on target cells.

In summary, here we show for the first time that endogenous opioid peptides inhibit the oestradiol-induced proliferation of human normal myometrial cells in culture. This opioid effect is mediated by a direct receptor mechanism of action, where the μ opiate receptor subtype is involved dominantly. ENK inhibited the oestradiol-driven decrease of proliferation and increase of size of pure smooth muscle cells, but in a naloxone-insensitive way, possibly bypassing the classical opiate receptors. The effects observed are within the physiological concen-

tration ranges. Based on our results, a novel physiological inhibitory role of endogenous opioid peptides could be suggested in the regulation of normal human uterine growth with a direct local mechanism of action. Stromal cells play an important role in the paracrine regulation of the development of the full trophic response of human uterus to oestradiol. This novel cross-talk between the opioid peptide and oestradiol signalling systems in human uterus may play an important role in physiological regulation during the menstrual cycle and pregnancy. Studies on the proliferation–inhibitory role of endogenous opioid peptides may even lead to a better understanding of the pathomechanism of oestrogen-related human uterine disorders, e.g. leiomyoma and endometrial cancer.

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