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Original article

The physiological role of β-endorphin in porcine ovarian follicles

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Abstract — β-Endorphin-like immunoreactivity (β-END-LI) was measured by radioimmunoassay in porcine ovarian follicular fluid (FF) from small, medium and large follicles throughout the oestrous cycle. The concentration of β-END-LI in FF from small follicles collected on days 1–5 of the cycle was at least tenfold higher than in the fluid from any other follicles independently from their size and the period of the cycle. The level of β-END-LI in small follicles on days 6–10 was drastically decreased. Subsequently, on days 11–16 its concentration was enhanced and reduced again in pre-ovulatory period of the cycle. Concentrations of β-END-LI in FF from medium follicles were relatively equal throughout the cycle (days 6–21). No significant differences in β-END-LI levels were found between small, medium and large follicles from days 17–21. However, β-END-LI concentrations in medium follicles on days 11–13 and 14–16 were statistically lower than those in small follicles. Moreover, the effects of FSH, prolactin (PRL), progesterone (P_4), testosterone (T) and 17 β-oestradiol (E_2) on β-END-LI release by granulosa cells (GCs) from large follicles and, on the other hand, the effects of the opioid agonist FK 33–824 alone or in combination with FSH, PRL or naloxone (NAL) on follicular steroidogenesis were studied. FSH drastically increased β-END-LI output in a dose-dependent fashion. This stimulatory effect of the gonadotrophin was inhibited by the highest dose of P_4 (10^{-5} M). The effect of PRL and the steroids added to the cultures on β-END-LI release was negligible. FSH- or PRL-induced P_4 secretion by GCs was essentially abolished by both FK 33–824 and NAL. However, androstenedione (A_4) and testosterone output by the cells was greatly potentiated by FK 33–824. In the presence of NAL, FSH or PRL, A_4 release stimulated by FK 33–824 was suppressed to the basal level. Secretion of E_2 was completely free from the influence of FK 33–824 or NAL; only oestrone (E_1) output was modulated by them in cultures where FSH or PRL was present. In conclusion, FSH appears to be the key regulator of β-END-LI secretion by porcine granulosa cells. Moreover, steroidogenesis in pig granulosa cells is modulated by opioid peptides acting both alone and by way of interaction with FSH or PRL.

opioid peptides / β-endorphin / porcine granulosa cells / steroid secretion

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Résumé — Le rôle physiologique de la β-endorphine dans les follicules ovariens de truie. Le niveau de β-endorphine immunoréactive (β-END-LI) a été mesuré avec la méthode radioimmunologique dans le liquide folliculaire provenant des petits, des grands et des moyens follicules de truie au cours de son cycle oestral. La concentration de β-END-LI dans le liquide folliculaire des petits follicules prélevés durant les 1er à 5e jours du cycle a été au moins 10 fois plus élevée en comparaison avec le liquide des autres follicules, indépendamment de leur taille ou de la phase du cycle. Le niveau de β-END-LI dans les petits follicules entre le 6e et le 10e jour a baissé d'une manière drastique. Ensuite, entre le 11e et le 16e jour, sa concentration a été élevée et de nouveau réduite durant le préoestrus dans le cycle. Les concentrations de β-END-LI dans le liquide folliculaire des follicules moyens ont été relativement égales durant les différents moments du cycle (6e–21e jour). Il n'y a pas eu de différences significatives du niveau de β-END-LI des petits, moyens ou grands follicules entre le 17 e et le 21e jour. Néanmoins, les concentrations de β-END-LI dans les follicules moyens entre le 11e et le 13e et entre le 14e et le 16e jour ont été statistiquement plus basses en comparaison avec les petits follicules. En outre, l'influence de FSH, de prolactine (PRL), de progestérone (P_4), de testostérone (T) et de 17 β-oestradiol (E_2) sur la libération du β-END-LI par les cellules de granulosa (GCs) provenant des grands follicules a été étudiée ainsi que l'effet de l'agoniste des opioïdes FK 33–824 seul ou en combinaison avec FSH, PRL ou nalaxone (NAL) sur la stérydogenèse folliculaire. FSH, suivant la dose, augmente d'une façon drastique la sécrétion de β-END-LI. Cet effet stimulateur de la gonadotrophine a été inhibé par P_4 en dose très élevée (10^{-5} M). L'influence de PRL et des stéroïdes ajoutés aux cultures sur la sécrétion de β END-LI a été négligeable. La sécrétion de P_4 induite par FSH ou PRL a été essentiellement réduite aussi bien sur l'influence de FK 33-824 que sur celle de NAL. Néanmoins, la sécrétion d'androstendione (A_4) et de testostérone par les cellules de granulosa a été stimulée d'une façon significative par FK 33–824. En présence de NAL, FSH ou de PRL la libération de A_4 stimulée par FK 33–824 a été réduite au niveau de base. La sécrétion de E_2 a été complètement indépendante de l'effet de FK 33–824 ou celui de NAL ; uniquement la sécrétion d'oestrone a été modulée par les deux facteurs sur les cultures auxquelles FSH ou PRL ont été ajoutés. En résumant, FSH semble être le facteur clé de la régulation de la sécrétion de β-END-LI par les cellules de granulosa chez la truie. En outre, la stéroïdogenèse dans les cellules de granulosa de truie est modulée par les peptides opioïdes agissant seuls ou bien en interaction avec FSH ou PRL.

peptides opioïdes / β-endorphine / cellules de granulosa de truie / sécrétion des stéroïdes

1. INTRODUCTION

Endogenous opioid peptides have a widespread distribution in both the central nervous system and peripheral tissues, including the male and female reproductive organs. β-Endorphin, one of the well-known representatives of this group of factors, has been found in the corpora lutea (CL) of sheep [31] and cows [10], in granulosa and interstitial cells of rodents [34, 35], and in human ovary [3]. Met-enkephalin, another extensively examined opioid peptide, has been demonstrated in bovine, rabbit and human ovaries [6, 30, 41]. Peptides that belong to the dynorphin family have been identified in the CL of cows [10] and in granulosa, luteal and interstitial cells of rats [35]. Moreover, α-neoendorphin has been found in porcine follicular fluid [46]. Confirmation of local opioid precursor production in the ovaries has come by way of identification of mRNAs for proopiomelanocortin [21, 38, 44], proenkephalin [21, 38] and prodynorphin [7]. Ovarian production and secretion of opioids is probably controlled by gonadotrophins. It has been shown that hCG and PMSG significantly augment the release of β-endorphin by rat ovaries [25, 36]. There is also evidence that granulosa cell proopiomelanocortin mRNA is under hormonal regulation by gonadotrophins and androgens [38].

Previous studies have shown that opioids may affect ovarian function both directly and indirectly, through modulating pituitary

LH secretion. Reports concerning the influence of opioids on steroidogenesis in human granulosa cells [11], in rat luteal cells [25] and in bovine luteal cells [51] suggest that opioid peptides exert autocrine and/or paracrine effects in the ovary.

In general, currently available data pertaining to the opioid content and its role within ovarian follicles are fragmentary, particularly in relation to domestic animals. Thus, the present study was undertaken to examine:

1. The content of β-END-LI in porcine follicular fluid taken from small, medium and large follicles throughout the oestrous cycle.

2. The influence of FSH, PRL, progesterone, oestradiol, and testosterone on β-END-LI release by granulosa cells from pig large follicles.

3. The effects of the opioid agonist FK 33–824, which interacts with μ and δ opioid receptors [43, 53], and naloxone (blocker of opioid receptors) on secretion of progesterone, androstenedione, testosterone, oestradiol-17β, and oestrone by granulosa cells from large follicles.

2. MATERIALS AND METHODS

2.1. Materials

Naloxone, opioid agonist FK 33–824, porcine FSH, insulin, hydrocortisone, transferrin, progesterone, androstenedione, testosterone, oestradiol-17β, oestrone and 24-well culture plates were purchased from Sigma (St. Louis, MO, USA). Prolactin (30 IU·mg^{-1}) was isolated from porcine pituitaries and kindly provided by Prof. Kazimierz Kochman (The Kielanowski Institute of Animal Physiology and Nutrition, Jablonna, near Warsaw, Poland). Labelled hormones: (1,2,6,7-^{3}H) progesterone, (1,2,6,7 (N)-^{3}H) androst-4-ene-3,17 dione, (1,2,6,7-^{3}H) testosterone, (2,4,6,7-^{3}H) oestrone, and (2,4,6,7-^{3}H) oestradiol were from Amersham, UK. Antisera against β-endorphin and porcine β-endorphin were obtained from Peninsula Laboratories Inc. (Belmont, CA, USA). Eagle's medium and trypsin were products of the Laboratory of Sera and Vaccines (Lublin, Poland), BSA fraction V was from the Laboratory of Sera and Vaccines (Kraków, Poland), antibiotics were from Polfa (Poland) and trypan blue from Chemapol (Czech Republic).

2.2. Isolation and incubation of granulosa cells

Ovaries were harvested from locally slaughtered cross-bred pigs, placed in cold buffered physiological saline (PBS) with antibiotics and immediately transported to the laboratory. The stage of the oestrous cycle was determined with the help of tables published by Akins and Morrissette [2]. Granulosa cells were isolated from large follicles (diameter > 6 mm) without signs of atresia. Follicles were classified as non-atretic if they had extensive and very fine vascularization, a regular granulosa cell layer and no free-floating particles in the follicular fluid. The cells were aspirated by syringe and additionally washed out with a strong stream of media directed to the internal wall of the follicle. The cells were centrifuged (800 × *g* for 10 min) and washed twice in Eagle's medium enriched with BSA (5%), penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). The cells were counted using a haemocytometer and their viability (98%) was determined by 0.4% trypan blue dye exclusion. The cells were resuspended in the incubation medium: Eagle's medium containing BSA (5%) (fraction V), hydrocortisone (40 ng·mL^{-1}), insulin (2 μg·mL^{-1}), transferrin (5 μg·mL^{-1}), and antibiotics as above. Additionally, in the experiments examining the effects of FSH and steroids on β-END-LI release by GCs, bacitracin (28 μg·mL^{-1}) was added to the incubation media. Medium composition was chosen according to our earlier experiences and to

results reported by Wiesak et al. [52] and Baranao and Hammond [5]. Granulosa cells (10^6 viable cells/well) were cultured in 24-well culture plates for 24 h in serum free conditions in a humidified incubator (37 °C) gassed with 95% air and 5% CO_2. Incubations were carried out in duplicate, and all experiments were repeated eight to twelve times. Following incubation in the presence and absence of stimulants, the media were harvested, centrifuged ($800 \times g$ for 10 min) and the supernatants were collected and stored at –20 °C until RIA analyses. None of the treatments affected the viability of the cells determined by trypan blue day exclusion.

2.2.1. Experimental series I: the content of β-END-LI in follicular fluid

Porcine ovaries, harvested from locally slaughtered cross-bred pigs, were divided into six groups according to the day of the oestrous cycle: viz. 1–5, 6–10, 11–13, 14–16, and 17–21. Follicular fluid (FF) was aspirated from small (1–3 mm in diameter) follicles throughout the whole oestrous cycle, from medium (3–6 mm) follicles on days 6–21 and large (> 6 mm) follicles on days 17–21. We chose only nonatretic follicles, i.e. uniformly translucent and vascularized. Follicular fluid was taken from several follicles (6–8) of ten to eighteen sows and pooled into one sample. Subsequently, the samples were centrifuged to obtain clear fluid, which was frozen immediately after bacitracin addition (28 $\mu g \cdot mL^{-1}$). β-END-LI in unextracted follicular fluid was subsequently determined by radioimmunoassay in triplicate in three independent experiments.

2.2.2. Experimental series II: effects of FSH, PRL and steroids on β-END-LI secretion by granulosa cells

In these experiments FSH (1, 10, 50, 100 $ng \cdot mL^{-1}$), PRL (1, 10, 100 $ng \cdot mL^{-1}$), progesterone (10^{-9}, 10^{-7}, 10^{-5} M), oestradiol (10^{-9}, 10^{-7}, 10^{-5} M) and testosterone (10^{-9}, 10^{-7}, 10^{-5} M) alone or in combination were added to the incubation media. The medium of the control group did not contain FSH, PRL or mentioned above steroids.

2.2.3. Experimental series III: effects of FK 33-824 and NAL alone and in combination with FSH and PRL on steroid secretion by granulosa cells

In these experiments the cultured cells were divided into nine groups treated with FK 33–824 alone (10^{-9} M), NAL alone (10^{-5} M), FK 33–824 with NAL, FSH alone (100 $ng \cdot mL^{-1}$), FSH with FK 33-824 or NAL, PRL alone (100 $ng \cdot mL^{-1}$), and PRL with FK 33–824 or NAL, and the results were compared with those from the control group of cells. The dose of each factor was established in our preliminary dose-response experiments and on the basis of the resulting study [11].

2.3. Radioimmunoassays of steroid hormones

Progesterone and oestradiol-17β concentrations were determined according to the method of Hotchkiss et al. [18] modified by Kotwica [27], those of A_4 and E_1 as described by Dziadkowiec et al. [9] and those of T according to Kotwica and Williams [28]. Antibodies against E_1 (BSz/88/706) showed the following cross-reactivities (oestrone 100%): oestradiol-17β-propionate (0.00%), 5α-androstane-3,17 dione (0.02%), androstenedione (0.01%), epiandrosterone (0.01%), androsterone (0.00%), progesterone (0.00%), testosterone (0.00%), 5β-androstane-3,17-dione (0.00%), 5β-androstane-3α, 17β-diol (0.00%), 4 androstane-11β-ol 3,17-dione (0.00%), 5α-pregnan-3α-ol-20-one (0.00%), 5α-pregnan-3β-ol-20-one (0.00%), 4-pregnen-20α-ol-3-one (0.00%), 4-pregnen-20β-ol-3-one (0.00%).

Cross-reactivities of the antisera against A_4, T, E_2 have been published previously [8]. The specificity of the antibodies against P_4 has been reported by Dziadkowiec et al. [9].

Validity of the assays was confirmed by parallelism between the standard curves and a series of dilutions of control culture medium and randomly chosen samples.

Intra- and inter-assay coefficients of variation of the P_4, A_4, T, E_2 and E_1 assays were 5.52%, 3.20%, 2.19%, 4.14% and 3.14%, and 15.09%, 8.68%, 5.15%, 5.87% and 9.21%, respectively. The sensitivities of the assays for P_4, A_4, T, E_2 and E_1 were 22 pg·mL^{-1}, 4.5 pg·mL^{-1}, 3.9 pg·mL^{-1}, 4.15 pg·mL^{-1} and 2.37 pg·mL^{-1}, respectively.

2.4. Radioimmunoassay of β-END-LI

β-Endorphin-like immunoreactivity in follicular fluid and media was established by the RIA procedure previously described by Ostrowska et al. [40], with the modification described by Okrasa et al. [39], in which a second antibody against rabbit γ-globulin (produced in our Department) was used to separate free from bound labelled β-endorphin. The rabbit antiserum against β-endorphin exhibited equimolar cross-reactivity (100%) with β-endorphin and β-lipotropin. Porcine β-endorphin was used for iodination and standards.

Incubation media, because of their low content of β-END-LI, were lyophilised before the assay. Samples were then reconstituted with 200 μL of assay buffer. Serial dilutions of the samples showed parallelism with the standard curve.

The sensitivity of the assay and the intra- and inter-assay coefficients of variation were 20 pg·mL^{-1} (at 92% binding), 8.52% and 16.21%, respectively.

2.5. Statistical analysis

All data from 3–12 separate experiments, each in duplicate or triplicate, were analysed by one-way analysis of variance and Duncan's test and are presented as mean ± SEM.

3. RESULTS

3.1. Experimental series I: the content of β-END-LI in follicular fluid

β-END-LI was detected in follicular fluid from each size of follicle taken from different phases of the oestrous cycle. The concentration of β-END-LI in FF from small follicles collected on days 1–5 of the cycle (4205 ± 26 pg·mL^{-1}) was at least ten-fold higher than in fluid from any other follicles independently from the period of the cycle. The level of β-END-LI in small follicles on days 6–10 had drastically decreased and subsequently raised significantly in a stepwise manner until days 14-16. The concentration of β-END-LI in FF from small follicles was decreased again in preovulatory period of the cycle (Fig. 1).

Concentrations of β-END-LI in FF from medium follicles did not show significant differences throughout the cycle (days 6–10, 11–13, 14–16, 17–21; 218 ± 8 pg·mL^{-1}, 219 ± 24 pg·mL^{-1}, 253 ± 11 pg·mL^{-1} and 268 ± 31 pg·mL^{-1}, respectively). No significant changes in β-END-LI levels were found between small, medium and large follicles from days 17-21 (262 ± 15 pg·mL^{-1}, 268 ± 31 pg·mL^{-1} and 321 ± 11 pg·mL^{-1}, respectively). However, β-END-LI concentrations showed essential differences ($P < 0.001$) on days 11–13 and 14–16, being higher in FF from small follicles than from medium ones (335 ± 14 pg·mL^{-1} vs. 219 ± 24 pg·mL^{-1} and 457 ± 28 pg·mL^{-1} vs. 253 ± 11 pg·mL^{-1}, respectively).

3.2. Experimental series II: effects of FSH, PRL and steroids on β-END-LI secretion by granulosa cells

Treatment of granulosa cells with FSH resulted in dramatic and dose-dependent

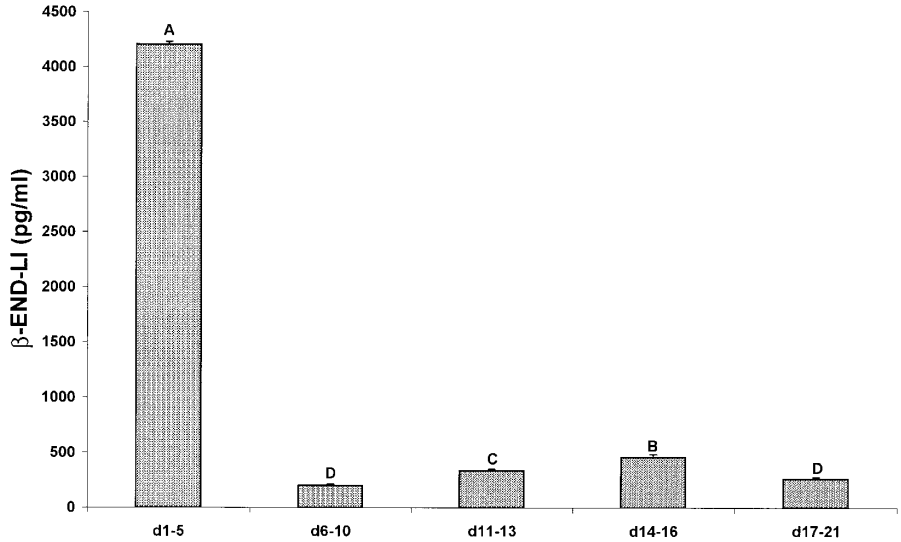

Figure 1. β-Endorphin-like immunoreactivity concentration in the follicular fluid from small follicles throughout the oestrous cycle. Bars with different superscripts are significantly different ($P < 0.001$). Results are means ± SEM of three replicates.

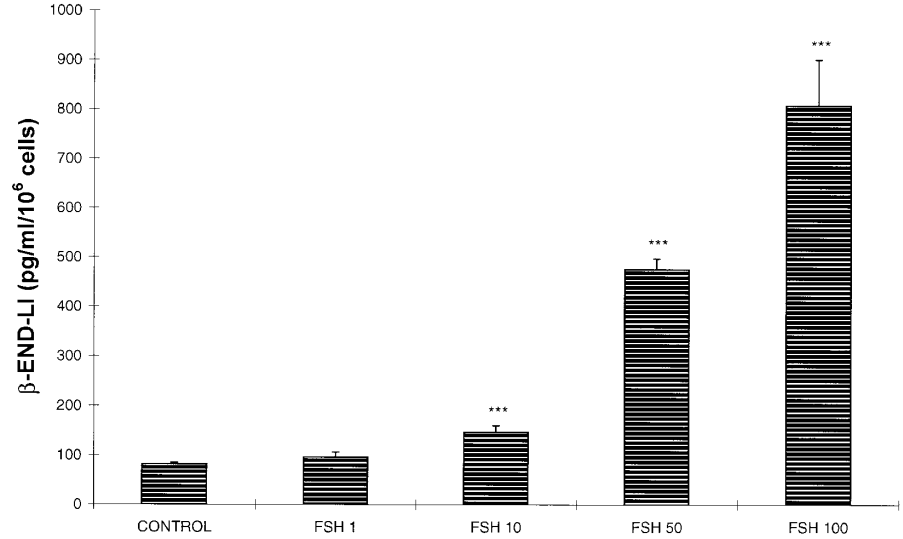

Figure 2. Dose-dependent effect of FSH (1–100 ng·mL^{-1}) on β-endorphin-like immunoreactivity secretion by cultured granulosa cells from large follicles. Results are means ± SEM of ten replicates. ***$P < 0.001$ compared with control.

potentiation of β-END-LI secretion compared with the control group (Fig. 2). The addition of FSH at a dose of 100 ng·mL^{-1} was followed by an eight-fold increase in β-END-LI release. The stimulatory effect of FSH was to some degree diminished ($P < 0.05$) by the highest dose (10^{-5} M) of P_4. Lower P_4 doses (10^{-7} and 10^{-9} M) did

not affect FSH-induced β-END-LI output (Fig. 3). Similarly, neither T nor E_2 influenced FSH-stimulated β-END-LI release. Basal (no FSH addition) β-END-LI secretion was not affected by any of the steroids used (data not shown). At the concentrations used (1–100 ng·mL^{-1}), PRL had a negligible effect on β-END-LI output (data not shown).

3.3. Experimental series III: effects of FK 33–824 and NAL alone and in combination with FSH and PRL on steroid secretion by granulosa cells

Neither FK 33–824 nor NAL affected P_4 secretion by porcine granulosa cells. Progesterone secretion was greatly potentiated by both FSH and PRL and the potentiation was completely abolished by both FK 33–824 and NAL (Fig. 4a).

In contrast, FK 33–824 significantly stimulated androstenedione release by the cells (Fig. 4b). In the presence of NAL, this effect was strongly reduced and A_4 secretion was suppressed to the basal level. Moreover, FSH and PRL also inhibited the effect of FK 33–824. It is also worth noting that co-treatment of the cells with PRL and NAL stimulated A_4 secretion compared with cells treated with PRL or NAL alone. The influence of the opioid agonist and antagonist on secretion of the second examined androgen, testosterone, was to some degree similar (Fig. 4c). Again, FK 33–824 greatly stimulated T release. However, this effect was slightly but not significantly inhibited by NAL, FSH or PRL. The addition of NAL together with PRL was followed by stimulation of T output in comparison with PRL alone.

The effects of FK 33–824 and NAL on oestrogen secretion were weakly expressed.

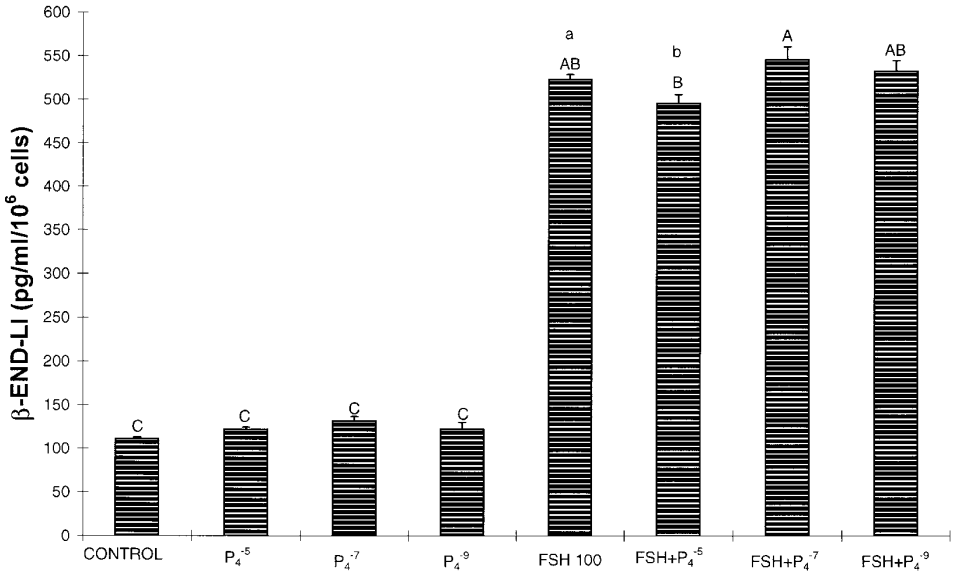

Figure 3. Effect of progesterone (10^{-9}–10^{-5} M) on basal and FSH-induced β-endorphin-like immunoreactivity secretion by cultured granulosa cells from large follicles. Results are means ± SEM of eight replicates. Bars with different superscripts are significantly different. Capital letters indicate $P < 0.01$ and small letters $P < 0.05$.

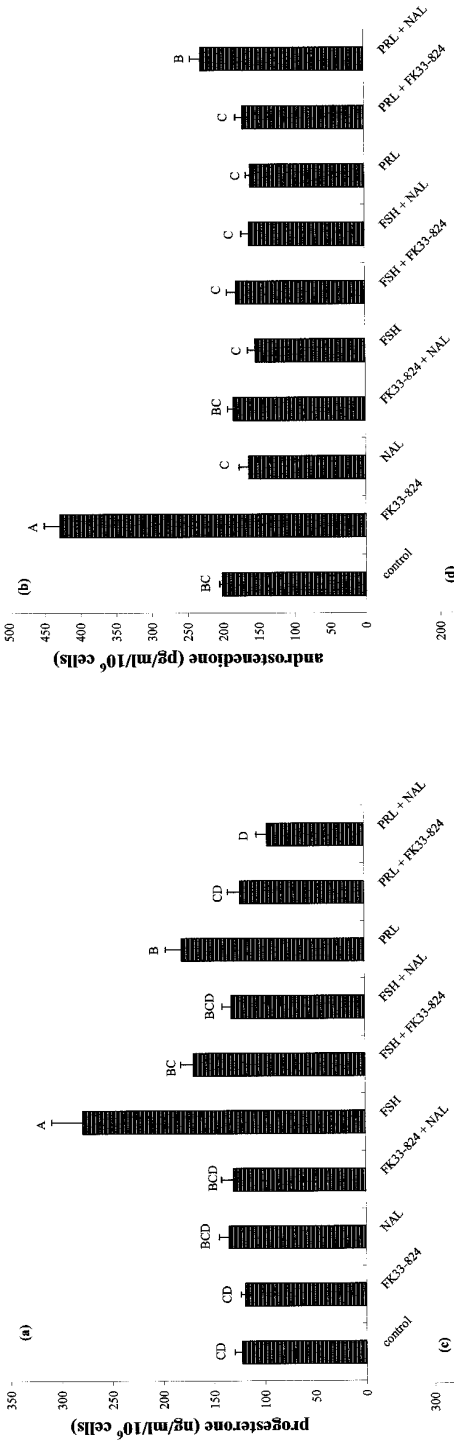

Figure 4. Effect of FK 33-824 alone or in combination with naloxone (NAL), FSH and PRL on (**a**) progesterone, (**b**) androstenedione, (**c**) testosterone and (**d**) oestrone secretion by cultured granulosa cells from large follicles. Results are means ± SEM of twelve replicates. Bars with different superscripts are significantly different ($P < 0.01$).

Secretion of E_2 was completely independent of these agents (data not shown); only E_1 release was to some degree modulated by them (Fig. 4d). The addition of NAL together with FSH caused reduction of E_1 secretion compared with the control group and the group in which FSH and FK 33–824 were added. Moreover, treatment of the cells with PRL in combination with FK 33–824 resulted in inhibition of E_1 release in comparison with FK 33–824 added alone.

4. DISCUSSION

In this study we assessed the level of β-END-LI in porcine follicular fluid and the effects of FSH, PRL and the most important ovarian steroid hormones on β-END-LI secretion by granulosa cells. Moreover, we examined the influence of the opioid agonist FK 33–824 added alone or together with NAL, FSH or PRL on steroid hormone release by porcine granulosa cells from large follicles. To our knowledge, up to now the content of immunoreactive β-endorphin in ovarian follicular fluid has been examined in two species: man [41] and rat [36]. In human follicular fluid the highest concentration of immunoreactive β-endorphin was found in the largest follicles. In the second case there were only small changes of immunoreactive β-endorphin content during the oestrous cycle. We observed the highest β-END-LI concentration in porcine FF from small follicles collected on days 1–5. In presented study we found that β-END-LI secretion by porcine granulosa cells is drastically enhanced in the presence of FSH. Thus, it seems that the main factor responsible for extremely high level of β-END-LI in small follicles taken in early luteal phase is just FSH. This finding is in good agreement with reports indicating that the highest plasma level of FSH takes place on day 3 of the pig oestrous cycle [42, 50]. Moreover, granulosa cells from small follicles have approximately 5 times as many FSH receptors as granulosa cells from large ones [29] and FSH receptor mRNA expression, which is strongly positive in granulosa cells of small follicles, declines essentially in the cells of large follicles [33]. Very low β-END-LI concentration in small follicles from days 17–21 is also in line with the lowest FSH level in blood plasma of pigs in follicular phase. In the case of medium follicles collected between days 6–21 of the cycle, we did not detect any relevant changes in β-END-LI level in relation to the phase of the cycle. However, on days 11–13 and 14–16 concentrations of β-END-LI in medium follicles were significantly lower in comparison with small ones collected in the same period. The observed differences could be caused by varied intrafollicular milieu in both types of follicles. Exemplary, the fluid from pig small follicles has much lower level of EGF [20] or follicle regulatory protein [47] and higher concentration of IGF-I [19] in comparison with medium follicles. EGF is known as a factor increasing FSH receptor number in porcine granulosa cells [37]. Opposite action demonstrates TGF_β, which reduces FSH binding to its receptors in porcine granulosa cells [13]. FSH receptor level in porcine granulosa cells can be also regulated by activin and inhibin. Activin, which was earlier isolated from follicular fluid of the pig [32, 49] is able to upregulate rat FSH receptor expression [16, 54]. In contrast with activin, precursor of α-inhibin was found to inhibit FSH binding to its receptors [45]. Inhibin is produced in vitro by granulosa cells from pig follicles [4] and its level in serum is the highest during the follicular phase [17]. It was suggested that medium and large follicles, containing the greatest amounts of free inhibin α subunit precursor, express the least activin [12, 26] and for these reasons such follicles might be less responsive to the tropic actions of FSH. Our experiments on porcine theca cells indicate that LH, PRL, P_4, T and E_2 did not affect β-END-LI release (Kaminski et al., unpublished observations). Thus, possibility arises that the noted

changes in follicular fluid β-END-LI levels are caused mainly by fluctuations in β-END-LI secretion by granulosa cells under influence of FSH and autocrine/paracrine factors. It is also worth noting that the regulation of follicular β-END-LI and another opioid peptide, α-neoendorphin, concentrations in porcine follicles seems to be quit different. While β-END-LI level is the highest in small follicles from early luteal phase, the highest α-neoendorphin concentration was found in porcine large preovulatory follicles [46].

In our experiments FSH appeared to be the major regulator of β-END-LI production by pig granulosa cells from large follicles, while the effects of PRL, progesterone, testosterone and oestradiol were negligible. Of the steroids administered together with FSH, only progesterone at the highest dose slightly modulated the stimulatory effect of the gonadotrophin on β-END-LI output. The present data are to a high degree consistent with those of previous studies showing that the level of β-END-LI in immature rat ovaries is four-fold higher after administration of PMSG [36]. Moreover, priming immature rats with PMSG, LH or androstenedione brought about many-fold increases in ovarian proopiomelanocortin mRNA levels [38].

In this study we have shown that the opioid agonist FK 33–824, and naloxone, markedly modulated steroid secretion by porcine granulosa cells. FK 33–824 interacting with μ and, to a lesser degree, δ receptors [43, 53] mimics the action of β-endorphin in this way. The effect of the opioid agonist and naloxone was observed both when they were added alone to the cultures and in combination with FSH or PRL. The latter was especially important as regards P_4 output. The stimulatory effect of FSH and PRL on P_4 release was abolished by FK 33–824 and NAL. This interdependence between FSH and PRL and the opioid agonist was reversed in relation to A_4 secretion. In this case the agonist-induced A_4 release was diminished in the presence of FSH or PRL. Moreover, the clear stimulatory effect of FK 33–824 on basal androstenedione and testosterone release observed in our experiment, coupled with the lack of an opioid agonist effect on basal, non-stimulated P_4 output, may suggest a direct influence of FK 33–824 on $P_{450c17\alpha}$. Interaction of opioid peptides with FSH, suggested by the present results, has also been indicated in a study by Facchinetti et al. [11]. They showed an increase in P_4 secretion by human granulosa cells after treatment of the cells with met-enkephalin and FSH compared with a group treated with FSH alone. The difference in relation to our findings, showing an inhibitory effect of FK 33–824 on FSH- and PRL-stimulated P_4 secretion, could be caused by differences between species and by the fact that the opioid agonist used in our study, rather than the opioid peptide, could interact differently with opioid receptors. On the other hand, our data are in line with recent results published by Gregoraszczuk and Slomczynska [14] indicating the existence of an inhibitory influence of β-endorphin on LH-stimulated P_4 secretion by pig granulosa cells. The suggested possibility of opioid and pituitary hormone interaction is also in accordance with previously published observations in porcine granulosa cells isolated from medium- or small-sized follicles [22, 23]. These findings imply the existence of cross-talk between signalling systems induced by opioids and FSH or PRL. It is possible that points of contact of signalling systems stimulated by FSH and opioid peptides are represented by adenyl cyclase and phosphoinositide-specific phospholipase C. Both enzymes are involved in the action of FK 33–824 on porcine small and large luteal cells [24] and porcine theca cells (Kaminski et al., unpublished information). However, because the mechanism of opioid peptide action inside ovarian cells is still only superficially understood, it is premature to

speculate on the mechanism of this cross-talk in detail.

The effects of FK 33–824 and naloxone on P_4 secretion observed in the present work were surprisingly similar. An explanation for this phenomenon may be connected to the different binding affinities of FK 33–824 and the opioid antagonist. While FK 33–824 binds to μ receptors and, in some measure, δ receptors, NAL at the concentration used blocks all types (μ, δ, κ) of opioid receptor. The occurrence of these types of opioid receptors in pig granulosa cells was previously indicated [15, 46]. Thus, κ receptors might be responsible for non-antagonistic action of NAL in comparison with the agonist effect on P_4 secretion by GCs. However, it is necessary to examine κ receptor ligand influence on steroidogenesis in porcine granulosa cells.

Our data might also suggest the existence of functional connections between different types of opioid receptor. Previously, this type of interaction has been found in other tissues. It is clear that μ and δ receptors can depend on each other [48]. Opioid peptides probably affect G_i protein connected to LH/hCG receptor action, causing a decrease of adenyl cyclase activity. The presence of such a mechanism was postulated by Kato et al. [25]. This hypothesis was confirmed in a study by Abramowitz and Campbell [1] that indicated an inhibitory effect of enkephalin amide (D-Ala^2, Met^5) on forskolin-activated adenyl cyclase of rabbit luteal cell membranes. However, a full explanation of opioid effects, including receptors and the intracellular messenger system, requires further study.

Taken together, our findings suggest that FSH is a key factor for efficient induction of β-END-LI secretion by porcine granulosa cells. Moreover, the results lead us to conclude that opioid peptides can have a meaningful effect on steroid hormone secretion by porcine granulosa cells. This effect is achieved at least in part by interaction of opioids with FSH and/or PRL.

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