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Opioid modulation of LH secretion by pig pituitary cells *in vitro*

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Summary. The effects of naloxone and β-endorphin on LH secretion by pig pituitary cells were studied in primary cultures. On Day 4 of culture, cells (10^5 seeded/well) were challenged with 10^{-9}, 10^{-8} or 10^{-7} M gonadotrophin-releasing hormone (GnRH), 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M-β-endorphin or 10^{-6} M-naloxone individually or in combinations. Secreted LH was measured at 4 h and 24 h after treatment and cellular content of LH was measured after 24 h. Basal LH secretion (control) was 23·5 ± 7·6 and 36·9 ± 10·3 ng/well at 4 h and 24 h, respectively. Relative to control at 4 h, 10^{-9}, 10^{-8} or 10^{-7} M-GnRH stimulated ($P < 0·05$) LH secretion 140%, 210% and 250%, respectively. At 24 h, LH secretion was increased ($P < 0·05$) by GnRH compared to control, but the dose–response to GnRH was absent. Naloxone increased ($P < 0·01$) LH secretion 166 ± 13% at 4 h and 141 ± 13% ($P < 0·06$) at 24 h. Secretion of LH after simultaneous addition of 10^{-8} M-GnRH plus naloxone was greater ($P < 0·01$) than after GnRH alone at 4 h but not at 24 h. β-Endorphin at 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M failed to alter basal LH secretion at 4 h but decreased secretion at 24 h, while cellular LH content was similar to control at 24 h. LH secretion after simultaneous addition of 10^{-7} M-GnRH and 10^{-7} M-β-endorphin was less ($P < 0·01$) than after GnRH at 4 h but not at 24 h, while 10^{-10} M-β-endorphin plus 10^{-9} M-GnRH failed to suppress LH secretion, compared to GnRH alone. These results indicate that endogenous opioids may directly modulate LH secretion at the level of the pituitary.

Keywords: opioid; LH; pituitary; pig

Introduction

Endogenous opioids may modulate luteinizing hormone (LH) secretion at the level of the central nervous system (CNS) and/or pituitary gland. Opioids may inhibit LH secretion by suppressing hypothalamic discharge of gonadotrophin-releasing hormone (GnRH; Wilkes & Yen, 1981; Blank & Roberts, 1982; Ching, 1983; Rasmussen *et al.*, 1989). Pituitary responsiveness to an exogenous challenge of GnRH is unaltered in animals treated with opioid agonists or antagonists (Cicero *et al.*, 1980; Ferin *et al.*, 1984). Moreover, some workers failed to demonstrate a direct opioid influence on pituitary LH secretion *in vitro* (Cicero *et al.*, 1979; Wiesner *et al.*, 1984). However, results of several recent studies indicate a direct in-vitro effect of opioid agonists on pituitary LH secretion in rats (Cacicedo & Franco, 1986; Blank *et al.*, 1986), cattle (Chao *et al.*, 1986) and sheep (Matteri & Moberg, 1985). Direct opioid modulation of pituitary gonadotrophs therefore remains a possibility. The opioid β-endorphin is found in the anterior pituitary gland (Smyth & Zakarian, 1980), as well as in hypophysial portal blood of rats (Wehrenberg *et al.*, 1982; Sarkar & Yen, 1985). Neurones containing β-endorphin have been identified within the hypothalamus of the pig

(Kineman *et al.*, 1989) and the role of opioids in modulating LH secretion has been demonstrated (Barb *et al.*, 1986, 1989).

The objective of this study was to determine whether LH secretion by dispersed anterior pituitary cells is modulated by a direct effect of opioids.

Materials and Methods

Preparation of pituitary cells. Two experiments were conducted utilizing 6 pituitary glands per experiment. Pituitary glands were aseptically removed from market weight gilts at slaughter. All subsequent procedures were performed under sterile conditions. The anterior lobes were dissected from each pituitary gland, washed three times in fresh Hanks' balanced salts solution (Sigma Chemical Co., St Louis, MO, USA) buffer (pH 7·4) containing 0·1 mg gentomycin/ml and finely minced with scissors. Each minced pituitary was transferred to a 50-ml plastic Erlenmeyer flask containing 20 ml 0·1 M-Hepes (Sigma) digestion buffer pH 7·4 [120 mM-NaCl; 50 mM-KCl; 5 mM-D-glucose; 1 mM-$CaCl_2$ H_20; 1·5% bovine serum albumin (BSA), Sigma] containing 3 mg collagenase Type I/ml (Sigma) and 12·5 μg DNase Type I/ml (2400 kU/mg; Sigma). Pituitaries were then incubated for 2 h at 37°C in a Dubnoff metabolic shaking bath with moderate agitation followed by centrifugation at 150 *g* for 10 min at room temperature. The supernatant was discarded and the cell pellet resuspended in 25 ml calcium-free Na_2EDTA (2 mg/ml) digestion buffer pH 7·4 containing 8 μg neuraminidase/ml (Sigma) and incubated for an additional 10 min at 37°C in a Dubnoff metabolic shaker with moderate agitation. The cell suspension was then filtered through 240 μm and 20 μm nylon screens to remove undigested tissue and cell aggregates and centrifuged at 150 *g* for 10 min at room temperature. The supernatant was discarded and the cell pellet washed twice by resuspending in 25 ml Dulbecco's modified Eagle's medium and Ham's Nutrient Mixture F-12 (DME/F_{12}; pH 7·4; 1·2 g $NaHCO_3$; 10 mg gentomycin; 5 mg amphotericin B/l; Sigma) and centrifuged at 150 *g* for 10 min at room temperature. Then the supernatant was discarded and the cell pellet resuspended in 5 ml culture medium [DME/F_{12} + 10% fetal bovine serum (FBS; Gibco, Grand Island, NY USA)] containing amphotericin and kanamycin (5 μg/ml; Sigma). Cell viability and number was determined by counting the number of cells excluding trypan blue on a haemocytometer (Tennant, 1964). Cells were diluted to 100 000 cells/ml with culture medium. Cells were plated at 1 ml cell suspension/well in a 24-well cluster plate and cultured at 37°C in an humidified atmosphere containing 5% CO_2. Culture medium was changed 48 h after seeding (day of seeding = Day 1 of culture) and experiments were performed on Day 4 of culture. There were either 4 or 5 wells per treatment per experiment.

Experimental protocol. At the beginning of each experiment, old medium was discarded, plates were rinsed twice with serum-free DME/F_{12} and pituitary cells were cultured in 1 ml fresh DME/F_{12} without serum but containing 10^{-9}, 10^{-8} or 10^{-7} M-GnRH, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M-β-endorphin or 10^{-6} M-naloxone alone or in combinations. A 0·1 ml sample of medium was harvested at 4 h and the remaining medium harvested at 24 h after addition of treatments. Cells were then solubilized with 0·2% Triton X-100 to recover intracellular LH. Both medium and cellular contents were assayed for LH (Kraeling *et al.*, 1982). The intra-assay and inter-assay coefficients of variation were 4·8% and 9·0% respectively.

Statistics. Data were converted to percentage of basal secretion before averaging to minimize differences between experiments. The data were then pooled across experiments. Basal secretion or content (control) was defined as the amount of LH per 10^5 cells secreted into the culture medium or LH cell content in the absence of GnRH. Data were then subjected to analysis of variance using the general linear model procedures of the Statistical Analysis System (SAS, 1982). Differences between treatment means were determined by least-squares contrasts (SAS, 1982).

Results

Basal LH secretion was 23·5 ± 7·5 ng/well (n = 9) and 36·9 ± 10·3 ng/well (n = 9) at 4 h and 24 h, respectively, while cell content for control averaged 47·1 ± 15·5 ng/well (n = 8). Relative to control at 4 h, 10^{-9}, 10^{-8} and 10^{-7} M-GnRH increased ($P < 0·05$) LH secretion 140%, 210% and 250%, respectively (Fig.1). However, at 24 h the LH dose–response to GnRH was absent but LH secretion was greater ($P < 0·05$) than for control (Fig. 1), while cellular LH content was suppressed ($P < 0·003$) for all doses compared to control at 24 h and averaged 70%, 49% and 77% (s.e.m. = 0·05%) for 10^{-9}, 10^{-8} and 10^{-7} M-GnRH, respectively. All doses of β-endorphin failed to alter LH secretion at 4 h except for 10^{-9} M-β-endorphin which increased ($P < 0·05$) LH secretion to 140 ± 10% of control (Fig. 2). In contrast, by 24 h of culture 10^{-10}, 10^{-9} and 10^{-7} M-β-endorphin inhibited LH secretion by 32 to 22% (Fig. 2), while cellular LH content for all doses was similar to control (data not shown). Naloxone (10^{-6} M) stimulated ($P < 0·01$) LH secretion 166 ± 13% while 10^{-8} M-GnRH elevated ($P < 0·01$) it to 206 ± 13% of control at 4 h (Fig. 3). Secretion of LH after

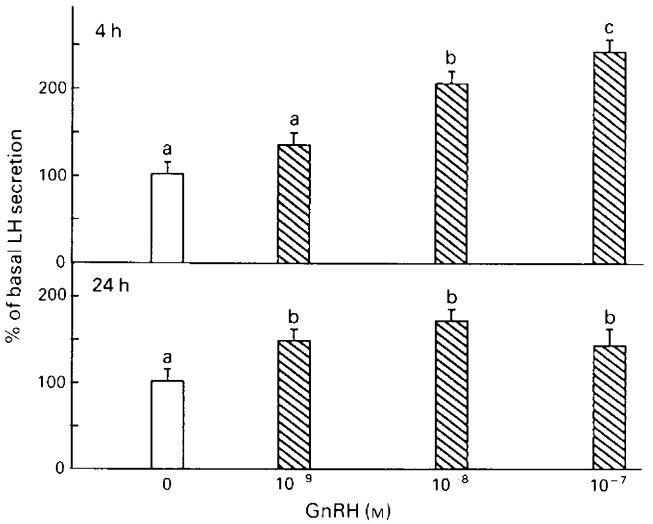

Fig. 1. Effects of 4-h and 24-h culture periods on basal and GnRH-stimulated release of LH in dispersed anterior pituitary cells of pigs. Values represent the mean ± s.e.m. (n = 8) except 0 (n = 9). Columns with different letters within a time period differ ($P < 0{\cdot}05$).

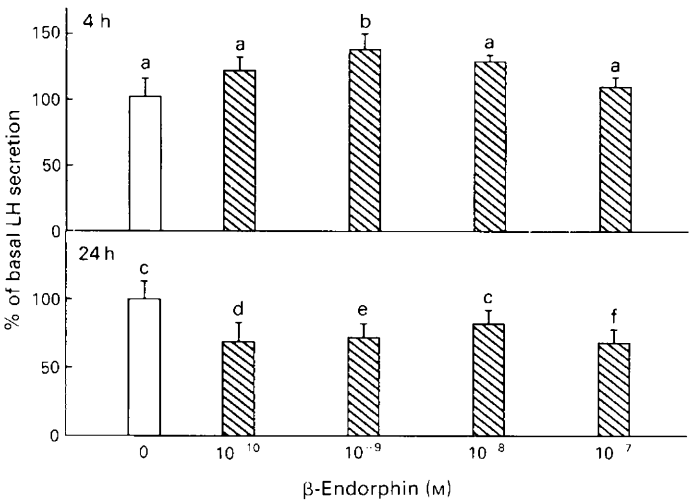

Fig. 2. Effects of β-endorphin on basal LH secretion during 4-h or 24-h culture periods. Values are mean ± s.e.m. (n = 8) except 0 (n = 9) and β-endorphin 10^{-10} M (n = 4). Columns with different letters within a time period differ from 0: $^{b}P < 0{\cdot}05$; $^{d}P < 0{\cdot}09$; $^{e}P < 0{\cdot}07$; $^{f}P < 0{\cdot}04$.

simultaneous addition of GnRH plus naloxone was greater ($P < 0{\cdot}01$) than GnRH alone at 4 h but this effect was not apparent at 24 h of culture (Fig. 3), while LH secretion for GnRH plus naloxone treatment was similar to that of control at 24 h. Cellular LH content was similar to control after addition of naloxone or naloxone plus GnRH (data not shown). Simultaneous addition of 10^{-6} M-naloxone and 10^{-10} M or 10^{-7} M-β-endorphin revealed an antagonism of LH releasing action between naloxone and β-endorphin after 4 h but not 24 h of culture (Fig. 3), while cellular LH content was not different from control (data not shown). Addition of 10^{-7} M-β-endorphin in combination with 10^{-7} M-GnRH suppressed ($P < 0{\cdot}01$) GnRH-induced LH secretion after 4 h but not

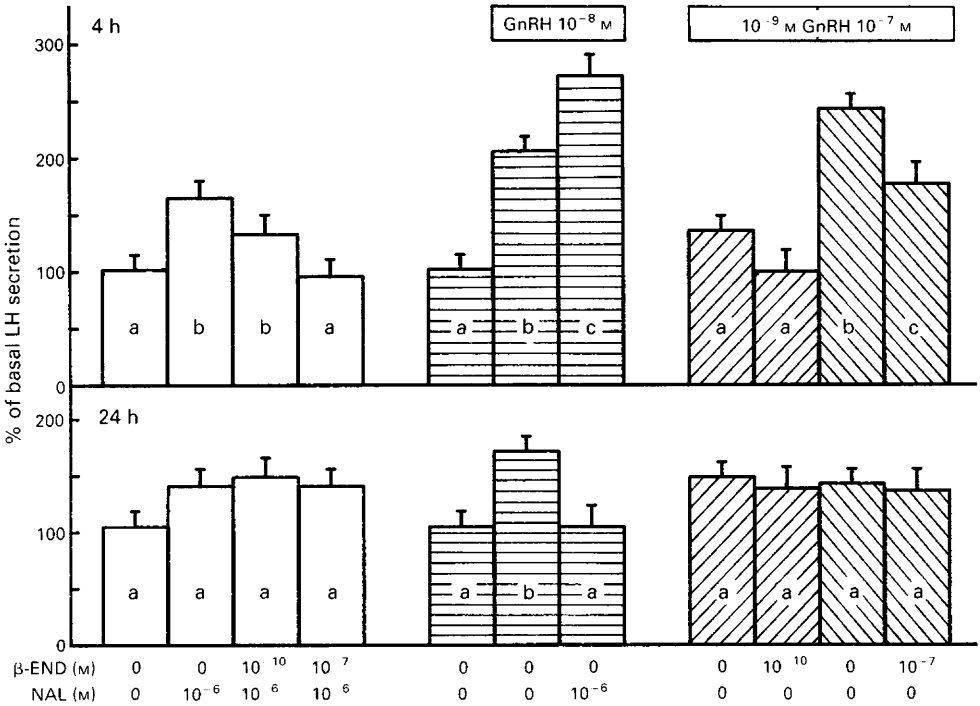

Fig. 3. The interaction of naloxone (NAL), β-endorphin (β-END) and GnRH on the secretion of LH from dispersed anterior pituitary cells cultured for 4-h or 24-h. Values are mean ± s.e.m.: 0 ($n = 9$), NAL ($n = 9$), GnRH ($n = 8$), GnRH + NAL ($n = 4$), β-END + NAL ($n = 7$), GnRH + β-END ($n = 4$). Columns with different letters within a time period differ; $^{a,b,c}P < 0{\cdot}01$.

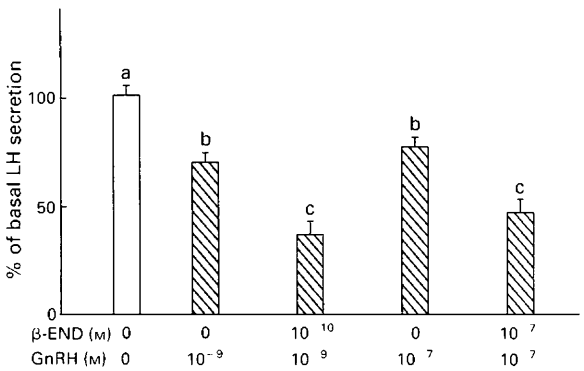

Fig. 4. The interaction of β-endorphin (β-END) with GnRH on cellular LH content relative to controls at 24 h. Values are mean ± s.e.m.: 0 ($n = 9$), GnRH 10^{-9} M ($n = 8$), GnRH 10^{-9} M + β-END 10^{-10} M ($n = 4$), GnRH 10^{-7} M ($n = 8$), GnRH 10^{-7} M + β-END 10^{-7} M ($n = 4$). Columns with different letters differ, $P < 0{\cdot}01$.

24 h of culture compared to GnRH alone (Fig. 3). Although 10^{-10} M-β-endorphin reduced the effect of 10^{-9} M-GnRH on LH secretion, this was not significant (Fig. 3). β-Endorphin in combinations with GnRH suppressed ($P < 0{\cdot}01$) cellular LH content compared to GnRH alone (Fig. 4).

Discussion

The present study demonstrates a direct effect of β-endorphin on LH secretion from anterior pituitary cells. The specificity of this effect is indicated by antagonism of β-endorphin inhibition of basal LH secretion by naloxone. Furthermore, increased basal and GnRH-induced LH secretion from cultured anterior pituitary cells exposed to naloxone suggests that opioids produced locally may play a role in modulating LH secretion.

The apparent similarity in LH concentrations between 4 h and 24 h may in part be due to several factors. First, when pituitary cells are continuously exposed to a secretagogue, i.e. GnRH, there is an initial release of LH, followed by a rapid decline in hormone secretion, reaching basal levels within several hours after treatment (Hopkins, 1977; Walker & Hopkins, 1978; Smith & Vale, 1981; Eddie *et al.*, 1982). Secondly, basal LH secretion continues to accumulate over time (Loumaye & Catt, 1983; Chao *et al.*, 1986). Therefore, when LH concentrations for treated cells are expressed as a percentage of control concentrations, 4 h concentrations appear to be greater than 24 h concentrations. In addition, this would account for the lack of a treatment effect of the various secretagogues at 24 h. We have no reasonable explanation for the similarity in LH secretion between control and naloxone + GnRH at 24 h. However, this is similar to results reported for LH secretion from bovine pituitary cells *in vitro* treated similarly (Chao *et al.*, 1986).

The β-endorphin-induced suppression of basal LH secretion was not apparent until 24 h after treatment. These results are consistent with those for which cultured pituitary cells of rats required 24 h exposure to β-endorphin (Cacicedo & Franco, 1986) or morphine (Blank *et al.*, 1986) to inhibit basal LH secretion. In contrast, methionine-enkephalin (Met-enk) inhibited LH secretion by 2 h after treatment in cultured bovine pituitary cells (Chao *et al.*, 1986). In addition, in sheep (Matteri & Moberg, 1985) γ-endorphin and human β-endorphin elevated LH secretion within 30 min of initiation of treatment. However, this effect was not demonstrable with ovine β-endorphin or Met-enk. It should be noted that, in the study by Matteri & Moberg (1985), cells were cultured in a perifusion system, while a static culture system was used by Cacicedo & Franco (1986), Blank *et al.* (1986) and Chao *et al.* (1986) and in the present study. Therefore, differences in LH secretion after opioid treatment between the studies may in part be related to the type of culture system used. Such delayed effects of opioids may be related to up-regulation of opioid receptor sites by exposure to the homologous ligand, i.e. β-endorphin, resulting in an increased sensitivity to the ligand and eventual inhibitory response (Yoburn *et al.*, 1988). Moreover, Blank *et al.* (1986) reported that, in the rat, exposure to morphine *in vitro* decreased LH secretion by 3 h in enriched gonadotroph cultures and after 24 h in unfractionated pituitary cells. The authors suggested that higher sensitivity of enriched gonadotrophs to morphine may be attributable to an increased availability of opioid receptor sites on the gonadotrophs due to absence of corticotrophs which produce opioids. An alternative hypothesis would be that opioid action may be mediated by modifying the GnRH receptor. In support of this idea, the stimulatory effects of human β-endorphin on LH secretion was suppressed by pretreatment of sheep pituitary cells *in vitro* with a GnRH receptor antagonist (Matteri & Moberg, 1985). However, this seems unlikely since treatment with opioid agonists failed to influence binding of GnRH to pituitary GnRH receptors in cattle (Moss *et al.*, 1985) and rats (Blank *et al.*, 1986).

In contrast to the delayed effect of β-endorphin on basal LH secretion, simultaneous addition of GnRH and β-endorphin reduced while GnRH and naloxone enhanced LH secretion by 4 h of culture. Similarly, Met-enk in cattle (Chao *et al.*, 1986) and human β-endorphin in sheep (Matteri & Moberg, 1985) reduced the GnRH-induced release of LH from pituitary cells in culture but naloxone failed to enhance basal LH secretion (Matteri & Moberg, 1985) or the GnRH-induced release of LH (Chao *et al.*, 1986). These results coupled with findings in the present study suggest species differences in the paracrine relationship between endogenous opioids and LH secretion at the level of the pituitary gland.

An increase in the transmembrane Ca^{2+} flux in pituitary gonadotrophs in response to GnRH is required for LH secretion (Huckle & Conn, 1989). Several reports have demonstrated that opioid

receptors are coupled to ion channels in neural tissue (Pepper & Henderson, 1980; Williams *et al.*, 1982; Werz & Macdonald, 1983) and stimulation of these receptors with opioid agonists decreased Ca^{2+} uptake (Ronai & Szekely, 1982). Opioid receptors have been dectected in the anterior pituitary gland (Lightmen *et al.*, 1983; Blank *et al.*, 1986). Therefore, opioid suppression of pituitary LH response to GnRH observed in the present study may be the result of altered transmembrane Ca^{2+} flux. Although the underlying mechanism(s) involved in β-endorphin-induced reduction in LH secretion remains to be determined, our data indicate that β-endorphin treatment depleted pools of releasable LH. Since β-endorphin suppressed basal and GnRH-induced LH secretion, cellular LH content should have been greater. However, this was not observed, suggesting that regeneration of releasable LH pools by synthesis of new LH was altered by β-endorphin.

The concentrations of β-endorphin used in the present study are comparable to intrapituitary and hypophysial portal blood concentrations. Intrapituitary β-endorphin concentrations are approximately 5×10^{-8} M in the rat (Rossier *et al.*, 1977) and $3–5 \times 10^{-9}$ M in the pig (Smyth & Zarkarian, 1980). Also, portal blood concentrations of β-endorphin may influence pituitary LH secretion, since portal blood concentrations of β-endorphin average 3×10^{-10} M to $1{\cdot}5 \times 10^{-9}$ M in the monkey (Wehrenberg *et al.*, 1982) and $2{\cdot}8 \times 10^{-9}$ M in the rat (Castanas *et al.*, 1984).

In summary, β-endorphin inhibited basal LH secretion *in vitro*. Stimulation of basal LH secretion by naloxone indicated inhibition of LH secretion by opioids endogenous to the anterior pituitary. These events appear to be mediated through opioid receptors, since stimulation by naloxone was antagonized by β-endorphin. The reduced LH response of cells treated simultaneously with GnRH and β-endorphin indicates that opioids may modulate the responsiveness of the pituitary gonadotroph to GnRH. Therefore, these results indicate that a paracrine relationship may exist between endogenous opioids and LH secretion at the level of the pituitary gland in the pig.

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