Interactions of ovarian steroids with pituitary adenylate cyclase-activating polypeptide and GnRH in anterior pituitary cells

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) releases LH and FSH from anterior pituitary cells. Although this effect is relatively weak, it has a strong sensitizing action on GnRH-induced gonadotropin secretion. Here we investigated the possibility that ovarian steroids, which are well-known modulators of LH secretion, interact with PACAP and GnRH in pituitary gonadotrophs. Rat pituitary cells were treated for 48 h with vehicle, 1 nmol/l estradiol, 1 nmol/l estradiol+100 nmol/l progesterone or 48 h with 1 nmol/l estradiol and 4 h with 100 nmol/l progesterone. The cells were stimulated for 3 h with 1 nmol/l GnRH or 100 nmol/l PACAP. Estradiol treatment alone enhanced basal as well as GnRH- or PACAP-stimulated LH secretion. LH release was facilitated by additional short-term progesterone treatment. Long-term treatment with estradiol and progesterone led to reduced LH responses to GnRH and PACAP. Neither treatment paradigms affected cAMP production. However, estradiol treatment led to enhanced cAMP accumulation in quiescent or GnRH-stimulated cells. PACAP-induced increases of cAMP production were inhibited by estradiol treatment.

After 7-h preincubation with 10 nmol/l PACAP, cells responded with enhanced LH secretion to GnRH stimulation. When steroid pretreatment was performed the responsiveness of gonadotrophs to low concentrations of GnRH was still increased. In contrast, at high concentrations of GnRH the sensitizing action of PACAP on agonist-induced LH secretion was lost in steroid-treated cells. There were no significant differences between the steroid treatment paradigms.

It is concluded that estradiol but not progesterone acts as a modulator of adenylyl cyclase in gonadotrophs. The stimulatory effect of estradiol is thought to be involved in its sensitizing action on agonist-induced LH secretion. The inhibitory effect of estradiol on PACAP-stimulated adenylyl cyclase activities seems to be responsible for the loss of its action to sensitize LH secretory responses to GnRH.

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was isolated from hypothalamic tissue by its ability to stimulate cAMP production in anterior pituitary cells (1). Two isoforms with 38 (PACAP 38) and 27 (PACAP 27) amino acid residues have been identified (2). The major isoform, PACAP 38, is found predominantly in the hypothalamus (3, 4). Although the physiological role of PACAP has not yet been clarified, it may act as a hypophysiotropic hormone. It has been shown to release a number of hormones including luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from anterior pituitary cells (1, 5, 6). Although its potency to release both gonadotropins is relatively low, it has a strong sensitizing effect on gonadotropin-releasing hormone (GnRH)-induced LH secretion (6, 7). This ability of PACAP might

be due to its stimulatory action on the cAMP–protein kinase A pathway in gonadotrophs since cAMP analogs and adenylyl cyclase activators enhance GnRH-stimulated LH secretion (8–10). If PACAP serves as a modulator of gonadotropin secretion it might interact with other hormones which are well-known modulators of gonadotropin secretion, such as ovarian steroids. Estradiol has a sensitizing effect on GnRH-induced LH secretion which is facilitated by progesterone (11–13). These steroid actions are essential for the initiation of the preovulatory LH surge (14). The prolonged exposure of the pituitary to progesterone in the luteal phase leads to suppressed gonadotropin secretion (15, 16). The combined actions of PACAP and gonadal steroids could be of physiological relevance for the cyclic regulation of gonadotropin secretion. In the present paper we examined the effects of PACAP 38, estradiol, progesterone and combinations of these hormones on

GnRH-stimulated LH secretion and cAMP production in cultured rat pituitary cells. These studies provide further information on the role of PACAP for gonadotropin secretion and steroid actions on adenyl cyclase.

Materials and methods

Pituitary cell preparation and culture conditions

Pituitary glands obtained from adult female Wistar rats (Winkelmann, Borcheln-Kirchborchen, Germany) at random stages of the estrous cycle were dispersed into single cells by controlled trypsinization as described previously (17). Dispersed pituitary cells were cultured in phenol red-free medium-199 (Biochrom, Berlin, Germany) containing Hank's salts supplemented with 1.4% sodium bicarbonate, 10% horse serum that had been pretreated with 2% charcoal (Norit A) and 0.2% Dextran T70 (Pharmacia, Uppsala, Sweden), 10 µg/ml streptomycin and 100 U/l penicillin at 37 °C under 5% CO₂/air and saturated humidity. Cells were grown on multiwell culture dishes (200 000 cells/well) and allowed to attach for 36–48 h before experiments were started. GnRH (Sigma, Deisenhofen, Germany) and PACAP 38 (Saxon, Hannover, Germany) were dissolved in phosphate-buffered serum (PBS) containing 0.1% bovine serum albumin. Stock solutions of estradiol and progesterone (Sigma) were prepared in ethanol. The final concentration of ethanol in the culture media was 0.2%.

Effects of estradiol, progesterone and PACAP 38 on basal and GnRH-induced LH secretion and cAMP release

In order to determine the effects of ovarian steroids on GnRH- and PACAP-stimulated LH secretion and cAMP release, pituitary cells were incubated for 48 h with vehicle (0.2% ethanol), 1 nmol/l estradiol, 1 nmol/l estradiol+100 nmol/l progesterone or pretreated for 48 h with 1 nmol/l estradiol and then treated for 4 h with 100 nmol/l progesterone. During the last 3 h of these treatment periods cells were incubated with or without 1 nmol/l GnRH or 100 nmol/l PACAP 38. Media were collected and analyzed for LH and cAMP.

Effects of PACAP on GnRH-induced LH secretion from steroid-treated cells

To test the effect of PACAP 38 on GnRH-induced LH secretion, pituitary cells were treated for 7 h with PACAP 38 and then stimulated with increasing concentrations of GnRH (0, 10⁻¹¹ to 10⁻⁶ mol/l). To elucidate the interactions of estradiol, progesterone and PACAP on GnRH-stimulated LH release, cells were incubated for 48 h with vehicle, 1 nmol/l estradiol,

1 nmol/l estradiol+100 nmol/l progesterone or pretreated for 48 h with 1 nmol/l estradiol and then treated for 4 h with 100 nmol/l progesterone. During the last 7 h the cells were incubated with or without 10 nmol/l PACAP. Then the cells were stimulated for 3 h with increasing concentrations of GnRH (0, 10⁻¹¹ to 10⁻⁶ mol/l). At the end of the stimulation periods with GnRH media were collected and analyzed for LH.

Radioimmunoassay and data analysis

The LH content of the samples was determined by radioimmunoassay, using the reference preparation RP-3 rat LH provided by the National Hormone and Pituitary Program (Rockville, MD, USA). The sensitivity of the assay was 312 ng/ml, the intra- and interassay variabilities were less than 10%. LH was expressed in ng/ml culture medium or in per cent of the LH release from vehicle-treated cells. The amount of cAMP in the media was determined by scintillation proximity assay (Biotrak SPA, Amersham International plc, Amersham, Bucks, UK). The sensitivity of the assay was 7 pg/ml, the intra- and interassay variabilities were less than 9%. The data are expressed in per cent of control (no PACAP, steroids or GnRH). All experiments were performed in triplicate and repeated three times. Statistically significant differences between treatments were determined by analysis of variance followed by a Newman–Keuls test using the InStat program (Graph Pad software, San Diego, CA, USA); *P*<0.05 was considered to be statistically significant. Dose–response curves were compared by the Allfit program (18).

Results

Effects of estradiol, progesterone and PACAP 38 on basal and GnRH-induced LH secretion and cAMP release

Long-term (48 h) treatment of pituitary cells with 1 nmol/l estradiol led to about twofold increases of basal LH release. This action was slightly enhanced by additional 4-h progesterone treatment while long-term treatment with estradiol and progesterone reversed the increase of LH release observed in estradiol-treated cells (Fig. 1). The release of cAMP into the culture medium was enhanced nearly threefold by estradiol treatment. This action was not influenced by additional progesterone treatment (Fig. 1). GnRH-induced LH secretion from pituitary cells was increased by 48-h estradiol incubation. This effect of estradiol was facilitated by the short-term progesterone treatment paradigm. The combined treatment with estradiol and progesterone led to reduced LH responses when compared with estradiol treatment alone (Fig. 2).

The modulatory actions of estradiol and progesterone on PACAP-stimulated LH secretion were similar (Fig. 3).

Figure 1 Effects of estradiol (E) and progesterone (P) on basal (upper) LH and (lower) cAMP release from cultured rat pituitary cells. Cells were treated for 48 h with vehicle (VEH; 0.2% ethanol), 1 nmol/l E, 1 nmol/l E+100 nmol/l P or for 48 h with 1 nmol/l E and a further 4 h with 100 nmol/l P. LH and cAMP were determined in the culture media at the end of the treatments during a 3-h period after washing the cells. Data are presented as the percentage (mean $\pm$ s.e.) of LH or cAMP secretion in VEH-treated cells (=100%). a, $P<0.05$ vs VEH; b, $P<0.05$ vs E.

Figure 2 Effects of estradiol (E) and progesterone (P) on GnRH-stimulated (upper) LH and (lower) cAMP release from cultured rat pituitary cells. Cells were treated with E and P as described in Fig. 1. LH and cAMP were determined in the media at the end of a 3-h stimulation period with 1 nmol/l GnRH. Data are expressed as the percentage (mean $\pm$ s.e.) of controls (CON; 100%; no steroid or GnRH treatments). a, $P<0.05$ vs CON; b, $P<0.05$ vs VEH; c, $P<0.05$ vs E; d, $P<0.05$ vs E 48 h+P 4 h.

PACAP itself led to a twofold stimulation of LH release. However, the magnitude of the sensitizing estradiol and progesterone effects was smaller than in GnRH-stimulated cells. GnRH was virtually without an effect on cAMP release (Fig. 2). This was also the case when the cells were incubated for prolonged periods (6 h) with the decapeptide (data not shown). As in non-stimulated cells, estradiol exerted a threefold increase of cAMP release that was not altered by short- or long-term progesterone treatment (Fig. 2).

PACAP induced a robust stimulatory action on cAMP release with 100-fold increases compared with controls (Fig. 3). In contrast to the action of estradiol in basal and GnRH-stimulated cAMP release, estradiol reduced the cAMP response to PACAP by about 50% (Fig. 3). Again, progesterone was without any action when compared with the effect of estradiol alone.

Effects of PACAP 38 on GnRH-induced LH secretion from steroid-treated cells

The data of the present study confirm our previous observations on steroid actions on GnRH-stimulated LH secretion. Long-term estradiol treatment has a stimulatory action on GnRH-induced LH secretion with enhanced maximal secretory responses and a shift in the EC_{50} from $5.6 \pm 1.3 \times 10^{-9}$ mol/l to $1.8 \pm 0.6 \times 10^{-9}$ mol/l (Fig. 4, Table 1). The additional short-term progesterone incubation led to increased sensitivity ($EC_{50} = 4.6 \pm 1.7 \times 10^{-10}$ mol/l). Combined treatment with estradiol and progesterone reduced the maximal response to GnRH and increased the EC_{50} to $2.0 \pm 0.4 \times 10^{-9}$ mol/l (Fig. 4, Table 1).

When the cells had been pretreated with PACAP a stimulatory action of the polypeptide on LH secretion was observed in the absence of GnRH (Fig. 5, Table 1).

Figure 3 Effects of estradiol (E) and progesterone (P) on PACAP-stimulated (upper) LH and (lower) cAMP release from cultured rat pituitary cells. Cells were treated with E and P as described in Fig. 1. LH and cAMP were determined in the media at the end of a 3-h stimulation period with 100 nmol/l PACAP 38. Data are expressed as the percentage (mean $\pm$ s.e.) of controls (CON; 100%; no steroid or PACAP treatments). a, $P<0.05$ vs CON; b, $P<0.05$ vs VEH; c, $P<0.05$ vs E; d, $P<0.05$ vs E 48h+P 4h.

Table 1 Effects of estradiol (E) and progesterone (P) on GnRH-stimulated LH secretion in PACAP-pretreated pituitary cells.

Treatment	Minimum	EC ₅₀	Maximum
No PACAP pretreatment			
Vehicle	4.5 $\pm$ 0.9	5.6 $\pm$ 1.3 $\times 10^{-9}$	38.7 $\pm$ 1.5
E 48h	6.5 $\pm$ 2.7	1.8 $\pm$ 0.6 $\times 10^{-9}$	75.7 $\pm$ 3.5
E 48h+P 4h	7.2 $\pm$ 3.8	4.6 $\pm$ 1.7 $\times 10^{-10}$	75.0 $\pm$ 3.3
E+P 48h	1.85 $\pm$ 1.0	2.0 $\pm$ 0.4 $\times 10^{-9}$	43.6 $\pm$ 1.3
PACAP pretreatment			
Vehicle	18.8 $\pm$ 1.2 ^a	2.0 $\pm$ 0.9 $\times 10^{-9}$	41.5 $\pm$ 1.6
E 48h	14.6 $\pm$ 2.7	5.5 $\pm$ 2.2 $\times 10^{-10}$	59.6 $\pm$ 2.4 ^a
E 48h+P 4h	15.5 $\pm$ 4.0	3.1 $\pm$ 1.7 $\times 10^{-10}$	61.3 $\pm$ 3.2 ^a
E+P 48h	10.4 $\pm$ 1.2 ^a	1.8 $\pm$ 1.0 $\times 10^{-9}$	34.8 $\pm$ 2.0

Analyses of LH dose-response curves by Allfit.

Minimum and maximum values are in ng LH/200 000 cells per ml. EC₅₀ values are in mol/l.

a, $P<0.05$ vs the corresponding steroid treatment in cells that were not pretreated with PACAP.

Figure 4 Effects of (upper) no pretreatment and (lower) PACAP pretreatment on sensitizing and desensitizing steroid actions on GnRH-stimulated LH secretion. Cells were treated with E and P as described in Fig. 1. Before the 3-h stimulation with GnRH cells were incubated for 7 h with or without 10 nmol/l PACAP 38. Vertical lines indicate EC₅₀ values.

Also, there was a leftward shift in the EC₅₀ in PACAP-pretreated GnRH-stimulated cells that did not receive any steroid treatments. The maximal response to GnRH remained unchanged (Fig. 5, Table 1). When we compared the steroid actions on GnRH-stimulated LH secretion from cells that were pretreated with PACAP with those that did not get such pretreatment, there were no significant changes in the EC₅₀ values. However, the maximal response was reduced in PACAP-pretreated cells. Furthermore, there were enhanced responses to low concentrations of GnRH as observed in cells that had not been pretreated with estradiol or progesterone (Figs 4 and 5).

Discussion

The findings in the present study show interactions of ovarian steroids with PACAP on LH secretion from

Figure 5 Comparison of dose–response curves to GnRH under different steroid treatment paradigms in the absence or presence of PACAP. Cells were treated with E and P as described in Fig. 1. Before the 3-h stimulation with GnRH cells were incubated for 7 h with or without 10 nmol/l PACAP 38. Vertical lines indicate EC_{50} values.

pituitary gonadotrophs. Secretory responses of these cells to PACAP are modulated by estradiol and progesterone in a similar manner as in GnRH-stimulated cells. Since GnRH is the primary regulator of LH and FSH and ovarian steroids serve as the most important modulators which exert the cyclic variations of gonadotropin release we have tested the possibility of whether PACAP could interfere with this process.

Recent studies have demonstrated that PACAP exerts a weak stimulatory action on LH secretion from cultured rat pituitary cells (1, 6, 19, 20). In addition, the polypeptide increases the sensitivity of pituitary gonadotrophs to GnRH, leading to a leftward shift of LH dose–response curves (7). The data of the present study have confirmed these findings. It has been claimed that PACAP might act as a hypophysiotropic factor and influence several functions of gonadotrophs. These include exocytosis of LH and FSH as well as gene expression of the gonadotropin subunits (6, 7, 21, 22). Presumably these PACAP actions are mediated via type I

receptors which have been shown to be coupled to adenylate cyclase and phospholipase C (22–24). The ability of PACAP to activate the cAMP–protein kinase A pathway is largely responsible for its sensitizing action on GnRH-induced LH secretion (for review see 25). We have shown that this effect is lost at high concentrations of GnRH. This might be due to the inhibitory action of GnRH on PACAP-induced cAMP production.

Furthermore, estradiol has been described as activating the cAMP–protein kinase A pathway. Such activity could influence PACAP and GnRH effects on gonadotroph functions. In the present study, estradiol was found to induce cAMP formation in the absence or presence of GnRH. This steroid effect could partially explain its sensitizing action on LH secretory responses of gonadotrophs as it has been demonstrated that elevation of cAMP in these cells leads to increased sensitivity to GnRH (8, 26). Other mechanisms such as GnRH receptor regulation are certainly involved (27–29).

Progesterone did not affect the increases of cAMP production that have been observed after estradiol incubation either in the short-term or in the long-term treatment paradigm. This finding demonstrates that progesterone modulation of GnRH-induced LH secretion does not involve a cAMP-dependent mechanism. Recent studies from our group have shown that progesterone exerts its inhibitory and stimulatory effects on gonadotropin secretion by acting on GnRH receptors and different pathways of GnRH signal transduction (30–34). In particular, the ability of progesterone to act as a bidirectional regulator of agonist-induced Ca^{2+} signals in gonadotrophs seems to be important for the modulation of secretory responses of these cells (31, 35). When PACAP was used to stimulate LH secretion from cultured rat pituitary cells that had been exposed to the different steroid treatment paradigms we observed the same modulatory actions of estradiol and progesterone as in GnRH-stimulated cells. Since PACAP activates phospholipase C leading to inositol phosphate production and increases of free intracellular Ca^{2+} concentrations it seems to be likely that the steroids act on this pathway to induce the observed changes of secretory responses to the polypeptide. In accordance with this possibility, we have previously described that estradiol and progesterone modulate LH secretion from gonadotrophs in response to other agonists that activate phospholipase C such as endothelin (36). The fact that gonadal steroids exert stimulatory and inhibitory actions on PACAP-stimulated LH release might explain the controversial data of *in vivo* studies on the ability of PACAP to induce gonadotropin secretion (19, 37–39).

The robust action of PACAP on cAMP formation was inhibited by 48-h estradiol treatment by about 50%. This is in contrast to the stimulatory action of estradiol alone or in the presence of GnRH on cAMP production. The addition of progesterone did not alter the inhibitory effect of estradiol, indicating the lack of progesterone action on adenylyl cyclase. The inhibition of PACAP-stimulated cAMP production by estradiol might interfere with the action of the polypeptide on gonadotropin secretion. However, pretreatment of pituitary cells with estradiol did enhance but not inhibit the LH secretory response to PACAP. Thus the augmenting action of estradiol on PACAP-stimulated LH secretion seems to be mediated by influences on phospholipase C-dependent signal transduction. Such mechanisms might also be involved in the regulation of progesterone effects on PACAP-induced gonadotropin secretion since this steroid does not influence adenylyl cyclase activity.

If PACAP acts as a physiological regulator of gonadotropin secretion its role as a modulator may be more important than its ability to act as a releasing hormone. Therefore, we investigated the combined actions of ovarian steroids, which are well-characterized modulators of gonadotropin secretion, PACAP and GnRH in gonadotrophs. We assumed that PACAP might interfere with the established inhibitory and

stimulatory effects of estradiol and progesterone on GnRH-stimulated LH secretion. Therefore, in addition to the previously tested steroid treatment paradigms, we introduced a 7-h PACAP incubation. Interestingly, at lower concentrations of GnRH the augmenting effect of PACAP on LH secretion was observed independently of the additional estradiol or progesterone treatment. However, at higher concentrations of GnRH, LH secretion was reduced by estradiol incubation. This effect was also present when progesterone was added to the culture media. The stimulating effect of PACAP at lower concentrations of GnRH might be due to its combined action on adenylyl cyclase which leads to sensitization of gonadotrophs and its ability to induce gonadotropin secretion via activation of phospholipase C. The latter effect seems to be more important at lower concentrations of GnRH. At higher concentrations of GnRH, the effect of PACAP to increase cAMP formation leads to enhanced LH responses which are lost at maximal doses of GnRH in cells that were not treated with steroids. However, estradiol which has been shown to inhibit PACAP-induced cAMP production in this study, also inhibits GnRH-stimulated LH secretion from PACAP-pretreated cells. Thus the sensitizing effect of PACAP on pituitary gonadotrophs is influenced by estradiol depending on the concentration of GnRH: at low concentrations there is no alteration of the PACAP action, at intermediate concentration the sensitizing effect is lost and at high concentrations the presence of PACAP is inhibitory.

In conclusion, estradiol but not progesterone acts as a modulator of adenylyl cyclase in gonadotrophs. In quiescent cells it increases cAMP formation while it is an inhibitor of PACAP-induced activation of adenylyl cyclase. The former effect is at least in part involved in its sensitizing action on agonist-stimulated LH secretion. The latter seems to be responsible for the loss of the PACAP action to sensitize LH secretory responses to GnRH. The influence of the steroid milieu on PACAP effects in gonadotrophs indicates that PACAP might act differentially during female reproductive cycles. The exact role for the regulation of gonadotropin secretion remains to be determined.

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