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MODULATORY ACTIONS OF ESTRADIOL AND PROGESTERONE ON PHORBOL ESTER-STIMULATED LH SECRETION FROM CULTURED RAT PITUITARY CELLS*

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Summary—We compared the ability of estradiol and progesterone to modulate gonadotropin-releasing hormone (GnRH) and protein kinase C (PKC)-mediated luteinizing hormone (LH) secretion. Long-term (48 h) treatment of rat pituitary cells with 1 nM estradiol enhanced GnRH and phorbol ester (TPA)-stimulated LH secretion. This positive effect was facilitated by additional short-term (4 h) treatment with progesterone (100 nM). However, long-term progesterone treatment, which inhibited GnRH-stimulated LH secretion, did not influence TPA-stimulated gonadotropin release. These steroid actions occurred without an effect on the total amount of LH in the cell cultures (total LH = LH secreted + LH remaining in the cell) and neither the secretagogues nor the steroids altered total LH. Since GnRH or TPA-induced LH secretion depends on Ca^{2+} influx into the gonadotroph, we also analyzed the effects of estradiol and progesterone under physiological extracellular Ca^{2+} concentrations and in the absence of extracellular Ca^{2+}. The steroids were able to influence GnRH or TPA-induced LH secretion under both conditions. However, when TPA was used as stimulus in Ca^{2+}-deficient medium the relative changes induced by estradiol and progesterone were more pronounced, possibly indicating that the extracellular Ca^{2+}-independent component of PKC-mediated LH secretion is more important for the regulation of the steroid effects.

It is concluded that estradiol and progesterone might mediate their modulatory actions on GnRH-stimulated LH secretion via an influence on PKC. This effect can occur independently from *de novo* synthesis of LH and Ca^{2+} influx into gonadotrophs.

INTRODUCTION

Estradiol and progesterone modulate gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH) secretion from cultured rat pituitary cells. Long-term estradiol treatment enhances GnRH-stimulated LH secretion, an effect facilitated by additional short-term progesterone treatment [1–7]. In contrast, long-term progesterone treatment of estradiol-primed pituitary cells reduces GnRH-stimulated secretion [3, 8, 9]. Although these effects are well characterized the mechanisms mediating the steroid actions on GnRH-stimulated gonadotropin secretion are poorly understood. A number of recent studies suggests that steroid modulation of the GnRH receptor number as well as postreceptor mechanisms might be responsible for the changes in sensitivity to the agonist [10–17].

GnRH also activates polyphosphoinositide hydrolysis leading to the liberation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 releases calcium (Ca^{2+}) from intracellular stores a process which is followed by Ca^{2+} influx through voltage sensitive Ca^{2+} channels (VSCC) [18]. DG serves as an activator of protein kinase C (PKC) [19–21]. This Ca^{2+}-dependent enzyme has been suggested to be involved in GnRH-stimulated LH secretion and synthesis [20]. Phorbol esters induce LH secretion which can be inhibited or enhanced by short- and long-term estradiol treatment, respectively [13, 15]. There is evidence that estradiol is able to increase total PKC activity in rat pituitary cells, an effect that might be responsible for the stimulatory action of estradiol on gonadotropin secretion [22].

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In the present study we determined the effects of estradiol and progesterone on LH secretion stimulated by GnRH or TPA (a PKC-activating phorbol ester). We have also investigated whether these treatments influence *de novo* synthesis of LH as this might contribute to the steroid actions on secretagogue-stimulated release of the hormone. Since both stimuli require Ca^{2+} influx into gonadotrophs to elicit the full LH response we analyzed the actions of estradiol and progesterone on extracellular Ca^{2+}-dependent and independent components of GnRH and TPA-induced LH secretion [23].

EXPERIMENTAL

Pituitary cell preparation and culture conditions

Pituitary glands obtained from 3-month-old female Wistar rats (Winkelmann, Borchen-Kirchborchen, Germany) were used for the preparation of primary cell cultures [24]. Cells were cultivated on multiwell culture dishes (200,000 cells/well) in medium 199 (M199) without phenol red with Hank's salts and L-glutamine (Biochrom, Berlin, Germany) supplemented with 1.4 g sodium bicarbonate/l, 10 μg streptomycin/ml, 100 U penicillin/ml and 10% horse serum (Biochrom) pretreated with 2% charcoal (Norit A) and 0.2% Dextran T70 (Pharmacia, Uppsala, Sweden) [25]. The cell cultures were maintained in a water-saturated atmosphere of 95% air–5% CO_2 at 37° for 48 h before the experiments were started. During the stimulation periods with GnRH or TPA supplemented M199 was used as described above except that 0.1% bovine serum albumin (BSA) was added instead of horse serum and Ca^{2+}-deficient medium (4 nM Ca^{2+}) was employed when indicated.

Effects of estradiol and progesterone on total LH (intracellular + secreted LH)

To determine whether the steroids alone exert an effect on LH synthesis pituitary cells were incubated for 48 h with culture medium containing vehicle (V, 0.2% ethanol), 1 nM estradiol, 1 nM estradiol + 100 nM progesterone, or 1 nM estradiol for 48 h and 100 nM progesterone for 4 h (all Sigma, Deisenhofen, Germany). The steroids were renewed every 24 h. At the end of the incubation period media were collected and stored at −20°C before LH was determined by RIA. To quantify intracellular LH the cells were lysed by the addition of 1 ml phosphate buffered saline (PBS) containing 0.1% Triton X-100 and freezing at −70°C. After two further freeze–thaw cycles the suspension of disrupted cells was aspirated and remaining cells were scraped from the dishes with a rubber policeman [26]. The suspension was centrifuged and the supernatant was stored at −20°C before determination of LH content.

Effects of GnRH and TPA on total LH (intracellular + secreted LH)

Pituitary cells were incubated for 3, 6, or 9 h with medium containing GnRH (Sigma; 1 nM and 1 μM) or 12-*o*-tetradecanoylphorbol-13-acetate (Sigma; TPA; 1 and 10 nM). Control cultures received solvents of GnRH (0.1% PBS containing 0.1% BSA) or TPA (0.01% dimethylsulfoxide). At the end of the incubation periods media were aspirated to determine the amount of secreted LH. The cells were lysed as described above for measurement of intracellular LH.

Effects of estradiol and progesterone on GnRH- or TPA-stimulated LH secretion

Pituitary cells were treated for 48 h with vehicle (V, 0.2% ethanol), 1 nM estradiol, 1 nM estradiol + 100 nM progesterone or 48 h with 1 nM estradiol and 4 h with 100 nM progesterone. During the last 3 h of the indicated incubation periods the cells were stimulated with increasing concentrations of GnRH (10 pM–1 μM) or TPA (100 pM–100 nM). At the end of the stimulation period the medium was aspirated and analyzed for LH content. To investigate whether combined treatment of the cell cultures with the steroids and either secretagogue has an effect on LH synthesis, we performed experiments in which the cells were pretreated as described above and stimulated for 3 h with a submaximal and a maximal concentration of GnRH (1 nM, 1 μM) or TPA (1 nM, 10 nM). At the end of the stimulation period the cell cultures were analyzed for secreted and intracellular LH as described above.

To determine whether extracellular Ca^{2+}-dependent and independent components of GnRH- or TPA-induced LH secretion are influenced differentially by steroid treatment, we performed experiments with normal M199 and Ca^{2+}-deficient M199. After estradiol and progesterone treatment under physiological extracellular Ca^{2+} conditions the cells were washed extensively and then stimulated with GnRH

Table 1. Effects of estradiol and progesterone on total LH (LH secreted + LH intracellular) in rat pituitary cell cultures

	LHi	LHs	LHtotal
V	33 ± 5*	6 ± 2	40 ± 6
E 48 h	29 ± 4	11 ± 2	39 ± 7
E + P 48 h	32 ± 3	9 ± 3	40 ± 4
E 48 h + 4 h	26 ± 4	15 ± 2**	41 ± 4

Rat pituitary cells were incubated for 48 h with vehicle (V, 0.2% ethanol), 1 nM estradiol (E), 1 nM estradiol + 100 nM progesterone (P), or for 48 h with 1 nM estradiol and 4 h with 100 nM progesterone. At the end of the incubation periods LH was determined in the culture media (secreted LH, LHs) and after cell lysis (intracellular LH, LHi) as described in Experimental.

*Data are ng LH RP-2/ml ± SEM. **Indicate $P < 0.05$ vs 48 h.

(1 nM, 1 μM) or TPA (1 nM, 10 nM) in normal or Ca^{2+}-deficient medium.

RIA and data analysis

The LH content of the samples was determined by RIA using the reference preparation RP-2 rat LH (AFP-5666 C) provided by the National Pituitary Agency (Baltimore, MD, U.S.A.) [27]. The data from 3 to 7 experiments performed in triplicate each were pooled and expressed in terms of percentage of the respective control cultures (100%) or as absolute LH values. Statistical analysis was performed as follows: analysis of variance (ANOVA) was carried out after a Bartlett test had shown homogeneity of variances. Statistical significant differences between individual groups were determined by the Newman–Keuls test. If variances were not homogenous, data were analyzed using the Kruskall–Wallis test followed by a Nemenyi test for comparison of individual groups.

RESULTS

Effects of estradiol and progesterone on total LH

Incubation of rat pituitary cell cultures for 48 h with estradiol or estradiol + progesterone or with estradiol and then 4 h with progesterone did not influence total LH compared to control cultures. However, long-term estradiol or short-term progesterone treatment did cause a small increase in basal LH release (Table 1).

Effects of GnRH and TPA on total LH

Stimulation of pituitary cells with submaximal or maximal concentrations of GnRH or TPA caused dose- and time-dependent increases in LH secretion. However, the additional analysis of intracellular LH revealed no differences between the total amount of LH after any of these treatments even when prolonged (9 h) exposure to the stimuli was performed (Fig. 1).

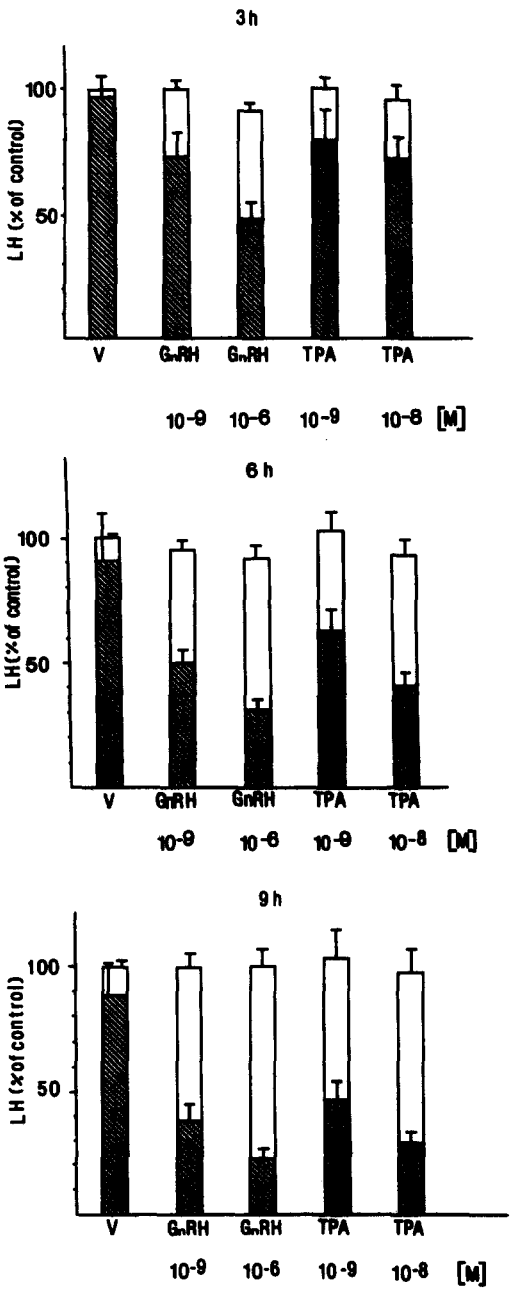

Fig. 1. Effects of GnRH or TPA treatment on the total amount of LH (intracellular + secreted LH) in rat pituitary cell cultures. Pituitary cells were treated to 3, 6, or 9 h with GnRH (1 nM, 1 μM) or TPA (1 nM, 10 nM). Total LH is depicted as the levels of LH in the culture media □ and in lysed cells ▨ at the end of the incubation with secretagogue. Data are presented as percentage of the total LH (sum of intracellular + secreted) of vehicle (V, no secretagogue, solvents of GnRH or TPA)-treated cells. No statistically significant differences were observed when total LH was compared between treatments. Total LH (ng RP-2/ml) in V-treated cell cultures was 73 ± 8, 70 ± 8, and 67 ± 8 at 3, 6, and 9 h, respectively.

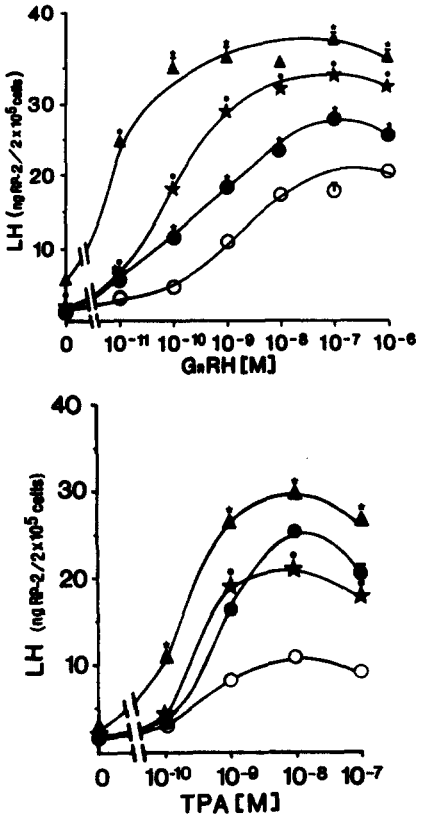

Fig. 2. Effects of estradiol (E) and progesterone (P) treatment on GnRH- or TPA-induced LH secretion from cultured rat pituitary cells. Cell cultures were treated for 48 h with vehicle (V, 0.2% ethanol, ○), or 1 nM estradiol (★), or 1 nM estradiol + 100 nM progesterone (●), or with 1 nM estradiol for 48 and 4 h with 100 nM progesterone (▲). During the last 3 h of the indicated incubation periods the cells were stimulated with GnRH (10 pM–1 μM, upper panel) or with TPA (100 pM–100 nM, lower panel). Representative data from 1 out of 3 independent experiments are shown. *Indicate $P < 0.05$ vs V, ⋆indicate $P < 0.05$ vs E 48 h. Statistical analysis was performed with data from three independent experiments.

Effects of estradiol and progesterone on GnRH- or TPA-induced LH secretion

Pituitary cells that were pretreated with estradiol showed enhanced LH responses to stimulation with increasing concentrations of GnRH or TPA. Coincubation with estradiol and progesterone for the same period resulted in a decrease of LH responses to GnRH compared to those after estradiol treatment. In contrast, there was no inhibitory effect of progesterone treatment when TPA was used as stimulus. Short-term progesterone treatment of estradiol-primed cells facilitated GnRH- and TPA-stimulated LH secretion. Maximal inhibitory or stimulatory effects of the steroids were observed at concentrations close to the ED_{50}s of GnRH or TPA, although the maximal releasing capacity of the cells to both stimuli were also clearly affected (Fig. 2).

Combined treatment of the cell cultures with estradiol, progesterone and the secretagogues had no effect on the total amount of LH either when GnRH or when TPA was employed at submaximal or maximal stimulatory concentrations, although the inhibitory and stimulatory effects of the steroids on LH secretion were present (Fig. 3).

When the effects of estradiol and progesterone on GnRH- or TPA-stimulated LH secretion were analyzed in cells that were cultured in Ca^{2+}-deficient medium there were no qualitative changes compared to the effects in normal medium, although the amount of LH secreted by cells maintained in Ca^{2+}-deficient medium was clearly smaller (Figs 4 and 5). In the absence of extracellular Ca^{2+} the LH responses to GnRH or TPA were reduced by 80 and 30%, respectively, which is consistent with data from previous studies [23]. Long-term estradiol and short-term progesterone treatment induced increased responsiveness to TPA stimulation. The relative changes compared to controls were more pronounced in the absence of extracellular Ca^{2+}. Under these conditions LH secretion reached or even exceeded the levels observed in cell cultures that were kept in regular M199 (Fig. 5).

DISCUSSION

We have evaluated the effects of estradiol and progesterone on GnRH- and TPA-stimulated LH secretion and have confirmed the previously demonstrated positive and negative effects of estradiol and progesterone on GnRH-stimulated gonadotropin secretion [1–9]. Furthermore it could be shown that TPA-induced LH release is enhanced by long-term estradiol and short-term progesterone treatment, while long-term progesterone treatment was ineffective, which is consistent with a recent report by Krey and Kamel [17]. Our data suggest that the facilitatory effects of the steroids on agonist-stimulated gonadotropin secretion might be mediated via an action on PKC.

We further examined whether the established estradiol and progesterone treatment paradigms are effective in changing the total amount of LH (total LH = amount secreted + amount remaining in the cell) in pituitary cell cultures. Such studies address the argument that the steroids primarily act on *de novo* synthesis of LH and

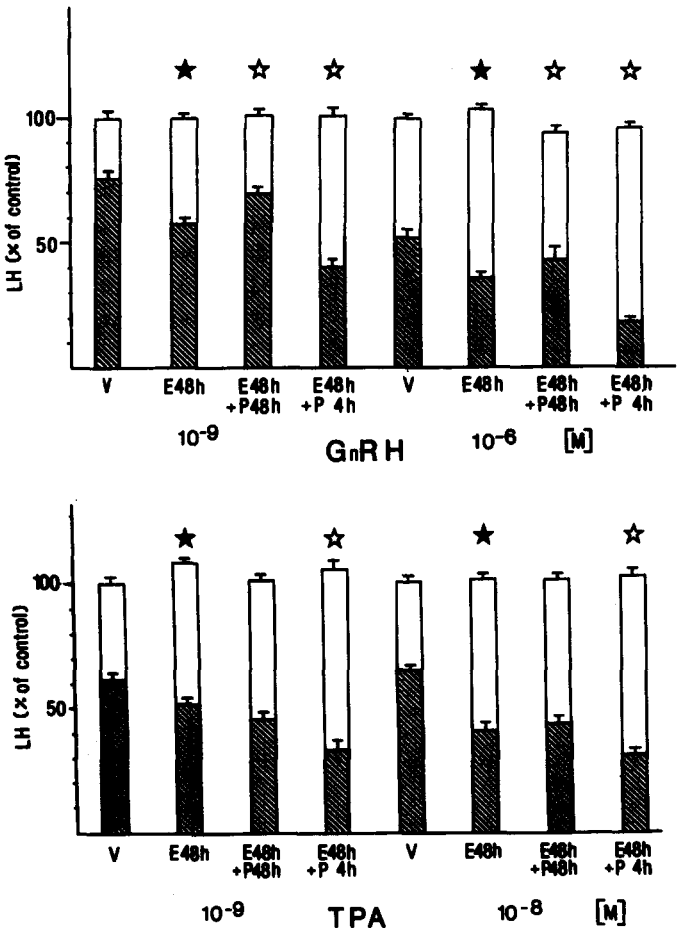

Fig. 3. Effects of estradiol (E) and progesterone (P) treatment on the total amount of LH (intracellular + secreted LH) in GnRH- or TPA- stimulated cell cultures. Steroid treatments were carried out as described in the legend to Fig. 2. During the last 3 h of the treatment periods the cells were stimulated with GnRH (1 nM, 1 μM; upper panel) or TPA (1 nM, 10 nM; lower panel). Total LH data are presented as percentage of the total amount of LH (intracellular ▨ + secreted □ = 100%). Estradiol and progesterone treatments are compared to respective vehicle (V)-treated cells (0.2% ethanol). Absolute values for total LH in V-treated cell cultures were 35 ± 2 at 1 nM and 40 ± 4 at 1 μM GnRH and 36 ± 2 at 1 nM and 36 ± 2 at 10 nM TPA. ★ Indicate $P < 0.05$ vs V, ☆ indicate $P < 0.05$ vs E 48 h of released LH.

that the modulated hormone release in response to GnRH or TPA reflects the steroid-induced changes on stored gonadotropins. While there were small stimulatory effects of long-term estradiol and short-term progesterone treatment on basal secretion, the total amount of LH was not influenced by any of the performed treatments. While GnRH and TPA had dose- and time-dependent stimulatory effects on LH secretion, the comparison between the total amounts of LH in non-stimulated and stimulated cell cultures did not reveal any differences during incubation periods that lasted up to 9 h. Other studies revealed conflicting results concerning the ability of GnRH and phorbol ester to induce gonadotropin synthesis [17, 20, 28–31]. Ramey *et al.* [30] have demonstrated that estradiol lowers the concentration of GnRH necessary to stimulate the biosynthetic response. The experiments performed here did not show such synergism between estradiol, progesterone and GnRH and TPA. Variations of the results from individual investigations might be due to different animal strains used, endocrine environment to which the pituitary was exposed, LH antisera, incubation periods, and culture conditions. Since the biosynthetic changes induced by estradiol which have been observed by others were relatively small, and the results of the experiments performed in the present study did not show any effect of steroid treatment alone or in combination with GnRH or TPA on LH biosynthesis, it can be concluded that the pathways responsible for stimulated hormone release are likely to be more important for the mechanism of the modulatory steroid actions.

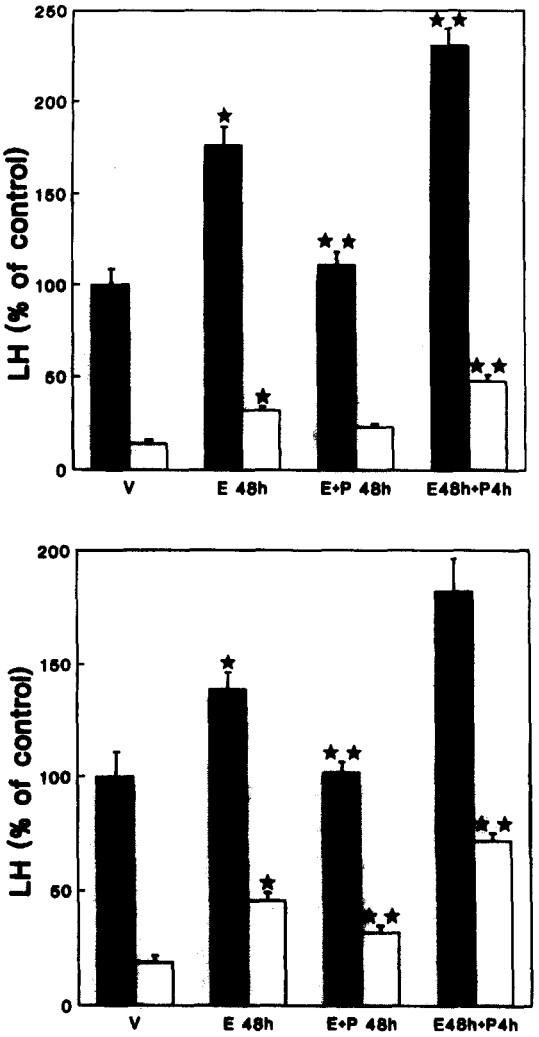

Fig. 4. Effects of estradiol (E) and progesterone (P) treatment on GnRH-induced LH secretion from cultured rat pituitary cells maintained in physiological Ca^{2+} or Ca^{2+}-deficient conditions. Steroid treatments were carried out as described in the legend to Fig. 2. During the last 3 h of the steroid treatment periods the cells were stimulated with GnRH (1 nM, upper panel; 1 μM, lower panel) in regular (■) or Ca^{2+}-deficient (□) medium. Data are presented as percentage of secreted LH from vehicle (V, 0.2% ethanol)-treated cells in regular Ca^{2+} medium (= 100%) stimulated by either 1 nM or 1 μM GnRH. The absolute LH value (ng RP-2/ml) corresponding to 100% is 27 ± 4 for 1 nM GnRH and 41 ± 6 for 10 nM GnRH. ★ Indicate $P < 0.05$ vs V; ★★ indicate $P < 0.05$ vs E 48 h.

There is evidence that ovarian steroids act on the GnRH signal transduction system and might mediate their effects on gonadotropin secretion via this mechanism. Estradiol and progesterone influence inositol phosphate production, agonist-induced cytosolic Ca^{2+} signals, and arachidonic acid-stimulated LH secretion [13, 14, 32, 33]. Our previous observation that short-term estradiol treatment inhibits TPA-induced LH secretion is extended by the present data which demonstrates positive actions of short-term progesterone and long-term estradiol treatment on PKC-mediated LH secretion [13]. These findings suggest that the PKC-dependent pathway of GnRH signal transduction can be regulated by the steroids. Furthermore recent studies have shown that estradiol has a positive effect on TPA-stimulated LH release and PKC activity with equal effects on membrane bound and soluble forms

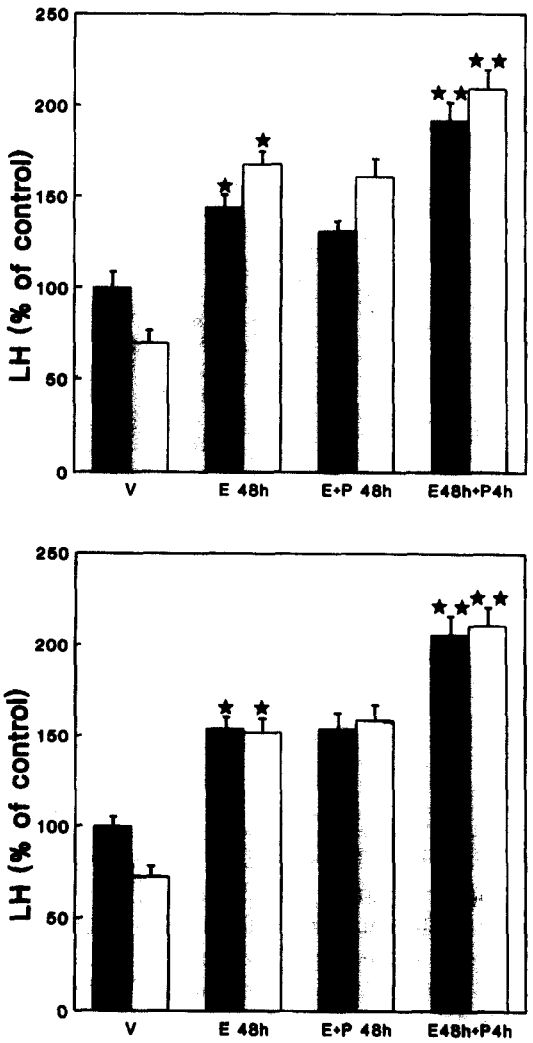

Fig. 5. Effects of estradiol (E) and progesterone (P) treatment on TPA-induced LH secretion from cultured rat pituitary cells maintained in physiological Ca^{2+} or Ca^{2+}-deficient conditions. Steroid treatments were carried out as described in the legend to Fig. 2. During the last 3 h of the steroid treatment periods the cells were stimulated with TPA 1 nM, upper panel; 10 nM, lower panel) in regular (■) or Ca^{2+}-deficient (□) medium. Data are presented as percentage of LH secretion from vehicle (V, 0.2% ethanol)-treated cells in regular or Ca^{2+} medium (= 100%) stimulated by either 1 or 10 nM TPA. The absolute LH value (ng RP-2/ml) corresponding to 100% is 27 ± 4 for 10 nM TPA and 31 ± 4 for 100 nM TPA. ★ Indicate $P < 0.05$ vs V; ★★ indicate $P < 0.05$ vs E 48 h.

of the enzyme [15, 22]. No enzyme activity studies have been performed with progesterone which could be of special interest as we observed a divergence of the GnRH and TPA paradigms. Although short-term progesterone treatment modulates GnRH- and TPA-induced LH secretion in a similar fashion, long-term progesterone treatment which inhibits GnRH-stimulated LH secretion does not alter the LH response to TPA. This divergence between the acute and chronic action of progesterone on PKC-mediated gonadotropin release implies that the two components of the biphasic progesterone action could be regulated differentially. Similar divergent actions of ovarian steroids have also been observed for arachidonic acid-induced LH secretion, which was modulated by acute estradiol or progesterone treatment although chronic treatment was ineffective [14]. Interestingly the data of the present study parallel the estradiol- and progesterone-induced changes of GnRH-receptor number in pituitary gonadotrophs. Long-term estradiol treatment increases the number of GnRH-receptors, an effect further enhanced by short-term progesterone treatment. In contrast, long-term progesterone administration does not decrease the receptor number below levels observed after chronic estradiol treatment [12]. It might be speculated that certain steroid actions on GnRH-stimulated LH secretion could be exerted by an influence on PKC-mediated GnRH-receptor regulation.

Although numerous reports have shown the ability of TPAs and membrane permeant DGs to activate PKC and induce LH secretion from pituitary gonadotrophs the role of this enzyme for GnRH-induced gonadotropin secretion is still a matter of controversy. Results of experiments with PKC-depleted cells show reduced responses to GnRH in most but not all studies [15, 20, 26, 34]. Recently a mechanism of PKC action has been proposed which was derived from the observation that TPA can induce cytosolic Ca^{2+} signals in rat pituitary cells [23]. Also the LH response to TPA has been shown to be dependent on extracellular Ca^{2+}. Cytosolic Ca^{2+} measurements in the presence of different Ca^{2+} channel blockers have revealed that TPA promotes Ca^{2+} entry into gonadotrophs through voltage-dependent dihydropyridine-sensitive Ca^{2+} channels and indicate that PKC can participate in GnRH-stimulated LH secretion via this mechanism [35]. But this cannot be the sole mechanism of PKC-mediated gonadotropin secretion since TPA also stimulates LH secretion kept in Ca^{2+}-deficient medium.

In this study we have examined the effects of estradiol and progesterone on extracellular Ca^{2+}-dependent and -independent components of GnRH and TPA action. GnRH-stimulated LH secretion was modulated by the steroids in a similar fashion under regular and Ca^{2+}-deficient conditions. These data demonstrate that pathways of GnRH signal transduction which operate through Ca^{2+} influx and those that are acting independently from this process are influenced by estradiol and progesterone. In TPA-stimulated cells the situation was different. In control cultures LH secretion was reduced by about 30% in Ca^{2+}-deficient medium. However, the modulatory effects of long-term estradiol and short-term progesterone treatment were more pronounced under these conditions than in cell cultures kept in regular medium. This could lead to the conclusion that the extracellular Ca^{2+}-independent component of TPA action is more important for the mechanism of steroid action. Recently we demonstrated that the cytosolic Ca^{2+} signal induced by TPA was amplified by short-term progesterone treatment, which implies that the extracellular Ca^{2+}-dependent component of TPA action is also modulated by the steroid [33]. The relative importance of both pathways of PKC-mediated LH secretion for the mechanism of steroid actions remains to be determined.

In conclusion we demonstrated that PKC-mediated LH secretion can be enhanced by estradiol and progesterone treatment of pituitary gonadotrophs. This effect does not necessarily require *de novo* synthesis of LH. The lack of an inhibitory effect of long-term progesterone treatment on TPA-stimulated LH secretion provides further evidence for the assumption that the modulatory effects of ovarian steroids on different GnRH signal transduction pathways are specific for the employed steroid and for the duration of treatment.

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