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CONTRACEPTIVE PROGESTINS AND GONADOTROPIN SECRETION *IN VITRO**

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Summary—In an *in vitro* bioassay using rat pituitary cell cultures the effect of contraceptive progestins was tested on basal and gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion *in vitro*. Progestins diminished gonadotropin release in pituitary cells stimulated with GnRH, but did not alter basal values. This inhibitory effect was dose dependent in a range of 10^{-10}–10^{-5} M tested and the inhibitory action of most of the progestins examined was more potent than that of progesterone. The maximal reduction of LH and FSH values was by 60% of GnRH-induced control levels. Progestins also caused a shift in sensitivity of cells to GnRh (10^{-12}–10^{-6} M). When time dependence was investigated, some progestins potentiated GnRH effect on gonadotropins in pituitary cell cultures pre-incubated for a short time (4 h) with steroids. More prolonged preincubations from 23 to 71 h resulted in a progressive suppression of LH and FSH response to GnRH (10^{-7} M). In order to examine intracellular effects, cells were pretreated with progestins and inositol phosphate metabolism was investigated. The data obtained in pituitary cells give evidence that polyphosphoinositide breakdown is potentially an early step in the action of GnRH on gonadotropin secretion by providing diacylglycerol and inositol phosphates. Addition of gonadotropin-releasing hormone to *myo*-2[^{3}H] inositol-prelabeled rat pituitary cells in primary culture evoked a dose-dependent increase of the accumulation of [^{3}H]inositol phosphates with a rise of inositol triphosphate, inositol diphosphate and inositol monophosphate within 1 min. Using one contraceptive progestin, gestoden, inositol phosphate production was inhibited by 80% compared to controls of GnRH-treated cells without the addition of steroids. The data obtained in this study suggest that this *in vitro* bioassay using rat pituitary cells is a useful tool in testing progestational compounds regarding their potency on gonadotropin release. In addition, these results show that one possible site of interference of progestins with GnRH-induced gonadotropin secretion may involve polyphosphoinositide breakdown.

INTRODUCTION

Clinical studies [1, 2] have already demonstrated that oral contraceptives dose dependently inhibit the release of gonadotropins in response to GnRH stimulation, although some investigators [3, 4] were unable to show this effect in women taking oral contraceptives.

Sex steroids have been known for some time to influence LH-secretion either by modulating GnRH release or by altering pituitary response to GnRH. These hormones may exert stimulatory or inhibitory effects on pituitary hormone secretion depending upon dosage, length of treatment and the hormonal environment.

Progestins have at least one property in common, i.e. they are all able to cause secretory transformation of an endometrium that has been proliferated by estrogens. As the effects of progestins are not exclusive to these substances, it is difficult to define accurately the physiological and pharmacological properties.

In vitro studies demonstrated that progesterone can potentiate or inhibit the sensitizing effect of estradiol depending on short- or long-term incubations, respectively [5–7]. In the luteal phase of ovariectomized ewes, progesterone decreased LH levels [8]. Some investigators [5, 6] reported, however, that progesterone added to incubation medium of rat pituitary cells on its own failed to affect the release of GnRH-stimulated LH and FSH. In pituitary cells originating from other species, such as sheep [9], the responsiveness of ovine pituitary cultures to GnRH was decreased.

The presence of progesterone receptors in cytosols and receptor–progesterone complex in nuclear fractions of the pituitary suggests that progesterone exerts its action on the pituitary itself.

In order to define a possible molecular site of progestin action in pituitary cells, one potential intracellular mechanism of GnRH-induced gonadotropin release was investigated. Until yet, several second messenger systems have been studied to explain the action of gonadotropin-releasing hormone (GnRH). The involvement of calcium in the receptor-mediated mechanism of GnRH is now generally accepted [10, 11]. However, GnRH-induced elevation of cyclic nucleotides cAMP [12, 13] and cGMP [14, 15] does not appear to be mediating the action of this decapeptide [16, 17]. Recent findings demonstrate the rapid changes in phospholipid metabolism in rat pituitary cells stimulated by GnRH with an increase of ^{32}P-labeling of phosphatidylinositol [18–20]. In other cell types it has

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been suggested that the hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol triphosphate directly releases calcium from intracellular stores [21]. Lately, evidence has been accumulated that GnRH stimulates the polyphosphoinositide breakdown in ^{32}P-prelabeled rat pituitary gonadotrophs [22, 23].

The aim of the present study was, first, to investigate the effect of progestins used in oral contraceptives on GnRH-stimulated gonadotropin release, and second, to determine whether polyphosphoinositide breakdown represents one possible intracellular site of progestin action.

EXPERIMENTAL

Culture conditions

Anterior pituitary cells of female Sprague–Dawley rats (250 g) were dispersed into a single cell suspension as described previously [19]. Cells (3×10^5 per culture dish) were incubated in 1 ml medium 199 "Earle" with 2.2 mg/ml $NaHCO_3$ (Seromed) supplemented with 10% Serum Plus (KC Biological), 800 μM L-glutamine (Seromed), 100 U penicillin and 100 mg streptomycin/ml (Seromed). All steroids were dissolved in 0.1% dimethyl sulfoxide (Serva) and added in the concentrations of 10^{-10}–10^{-5} M to each well. After 3 days in culture, the cells were washed twice and incubated in medium 199 "Earle" with 20 mM HEPES (Seromed), supplemented with 0.85 mg/ml $NaHCO_3$, 800 μM L-glutamine (Seromed), 100 U penicillin and 100 mg streptomycin/ml (Seromed) and 0.25% bovine albumin (Sigma). For stimulation of luteinizing hormone (LH) release, synthetic gonadotropin releasing hormone (GnRH, Peninsula Laboratories Inc.) was added to the medium in a final dilution of 10^{-7} M. After 4 h of incubation, the supernatant was saved for radioimmunoassay of LH. Cultures with 0.1% dimethyl sulfoxide were used as control, which were not different from controls without the solvent.

Progestins and gonadotropin secretion

The effect of contraceptive progestins on LH and FSH was investigated by using various conditions. To determine time dependence of steroid action, cells were preincubated with progestins for 4–72 h before being challenged with GnRH. Dose-dependent effect was studied using a 72-h incubation period and steroid concentrations were varied from 10^{-10} to 10^{-5} M. To determine a shift in sensitivity of cells to GnRH, progestins (10^{-5} M) were added to pituicytes for 72 h and cells were stimulated by GnRH (10^{-12}–10^{-6} M).

The following steroids were studied: progesterone, cyproterone, cyproterone acetate, chlormadinone, chlormadinone acetate, ethynodiol acetate, norgestimate, gestoden, norethinodrel, levonorgestrel, 3-keto-desogestrel, norethisterone, norethisterone acetate, norgestrel, lynestrenol and desogestrel.

Progestins and inositol phosphate production

Two million cells/well were incubated for 4 days in Medium 199 Earle (modified, Seromed, West Berlin). On day 2 of incubation, 12×10^6 cpm *myo*-2[^{3}H]inositol (10–20 Ci/mmol; Amersham) was added to each well. On day 4, medium was removed and replaced by stimulation medium (Medium 199 Earle, 20 mM HEPES, 0.01% BSA). Ten min prior to stimulation 10 mM, $LiCl_2$ was added to prevent inositol phosphate breakdown. Then GnRH was added at a final concentration of 10^{-7} M. Reaction was stopped by placing wells on dry ice. Medium was removed and saved for measurement of LH by radioimmunoassay. Immediately thereafter, ice-cold methanol–6 N HCl (1:200) solution was added (2 ml/well) and transferred to tubes with chloroform (1 ml) and water (1.8 ml). Cells were scraped off using a rubber policeman. The extracts were sonified (for 5 min) and centrifuged (3000 **g** for 10 min). The upper water-soluble phase containing inositol phosphates was separated from the lower phase containing the lipid fraction (polyphosphoinositides). This method is a modification of the extraction procedure described by Berridge *et al.*[24]. The radiolabeled inositol phosphates in the aqueous phase were determined by anion exchange chromatography on columns (Pasteur pipettes) containing AG_1-$\times 8$ resins (BioRad, 200–400 mesh, formate form). Free inositol was eluted in the flow-through of distilled water and glycerophosphate by disodium tetraborate 5 mM–sodium formate 60 mM. Then sequential washes with 0.1 M formic acid containing 0.2, 0.4 and 1.0 M ammonium formate progressively eluted IP_1, IP_2 and IP_3. Five-ml fractions were collected and measured after addition of 10 ml scintillation fluid (Amersham) for myo-2-[^{3}H]inositol. Elution curve is shown in Fig. 7.

Standard curves have been established using radiolabeled inositol phosphates. Each data-point consists of three independent control values and shows one out of two experiments.

RESULTS

Time-dependent inhibition of gonadotropin secretion

Contraceptive progestins and progesterone showed a time-dependent suppression of GnRH-stimulated LH release in rat pituitary cells, using the following progestational compounds: progesterone, ethynodiol diacetate, norgestrel, levonorgestrel, norethisterone, norethisterone acetate, gestoden, 3-keto-desogestrel, desogestrel, norgestimate, chlormadinone, chlormadinone acetate, lynestrenol, cyproterone and cyproterone acetate.

Figure 1 demonstrates an example of time-

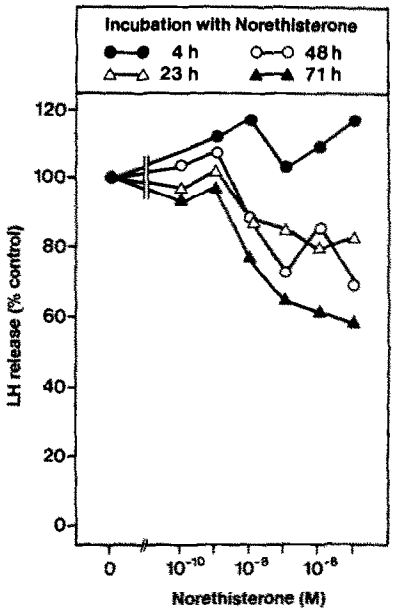

Fig. 1. Time- and dose-dependent effect of norethisterone on GnRH (10^{-7} M)-stimulated LH release (100%). The concentration of norethisterone ranged from 10^{-10} to 10^{-5} M. Incubations were carried out at 4, 23, 48 and 71 h in media containing norethisterone. Following these preincubation times, cells were washed twice and stimulated by GnRH (10^{-7}). Controls contained 0.1% dimethyl sulfoxide (100%).

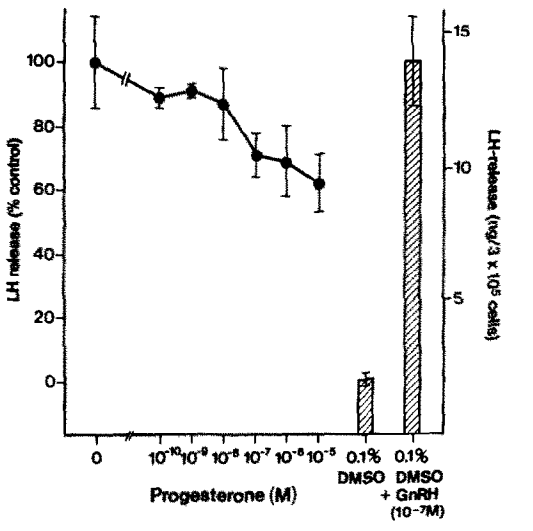

Fig. 2. Inhibition of GnRH-induced LH release by progesterone. Controls contained 0.1% dimethyl sulfoxide. Following 72 h of progesterone pretreatment, cells were washed twice and incubated with GnRH (10^{-7} M) for 4 h. The difference between GnRH-stimulated and basal LH values is taken as 100%.

dependent inhibition of LH-release by norethisterone representative of other steroids. Short-term incubation (4 h) showed a slight enhancement of gonadotropin release. With increasing preincubation time (23–71 h), the secretion of LH was progressively inhibited. Maximal inhibition was achieved at 71 h with 10^{-5} M progestin.

Dose-dependent inhibition of gonadotropin release

Increasing progestin concentration caused a dose-dependent inhibition of GnRH (10^{-7} M) induced LH release, as shown, for example, for norethisterone (Fig. 1) and for progesterone (Fig. 2) in cells pretreated with steroid for 72 h. Results were calculated as per cent control. The difference between GnRH-stimulated and basal LH secretion was taken as 100%. All samples contained the solvent (0.1% dimethyl sulfoxide), which had no significant effect on cell response during the time examined.

The following values were determined for the inhibition of GnRH-stimulated LH secretion by 10^{-5} M progestins (expressed as % control = GnRH stimulation without steroid preincubation): lynestrenol (52%), 3-keto-desogestrel (51%), norgestrel (51%), norethisterone (50%), ethynodiol diacetate (50%), norgestimate (46%), gestoden (44%), chlormadinone acetate (43%), levonorgestrel (37%), norethisterone acetate (21%), desogestrel (15%), progesterone (15%), cyproterone acetate (9%) and cyproterone (8%).

Shift of GnRH-response curve of LH and FSH release by progestins

Progestins have been tested for their potency to shift the response curve of LH and FSH stimulated by GnRH (Figs 3–6). Control was defined as the response of pituitary cells to GnRH (10^{-12} to 10^{-6} M) by secreting LH and FSH. The effects of progestins were examined by preincubating cells with the steroids for 48 h using 10^{-5} M steroid concentrations. Progesterone had an inhibitory effect on LH release and no significant effect on FSH release (Fig. 3). In Figs 4–6 examples of progestins (norgestrel, norgestimate and gestoden) are shown for the effect of progestational compounds on GnRH-induced LH release. Almost all contraceptive progestins caused a shift of the GnRH-stimulated curve to the right and inhibited maximal cell response to GnRH. Cells pretreated with progestins are therefore less sensitive to GnRH stimulation. The action of progestins on LH was comparable to their effect on FSH secretion (Figs 4–6).

Half-maximal stimulation (ED_{50}) of LH release by GnRH was 3.2×10^{-10} M under the conditions examined. The ED_{50} value for GnRH response was altered by the following progestins: progesterone (8×10^{-10}), cyproterone (4×10^{-10}), cyproterone acetate (1.1×10^{-9}), chlormadinone acetate (1.6×10^{-9}), ethynodiol acetate (1.6×10^{-9}), norgestimate (1.6×10^{-9}), gestoden (1.8×10^{-9}), levonorgestrel (2×10^{-9}) 3-keto-desogestrel (2.5×10^{-9}), norethisterone (2.5×10^{-9}), norethisterone acetate (2.5×10^{-9}), lynestrenol (3.6×10^{-9}) and desogestrel (4.5×10^{-9}).

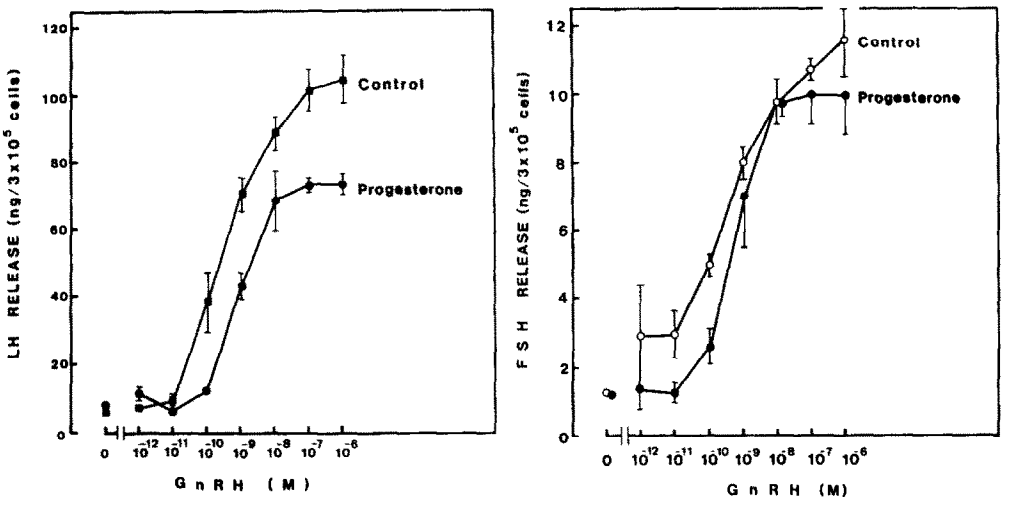

Fig. 3. Shift of GnRH-response curve of LH and FSH release by progesterone. Cells were pretreated by 10^{-5} M progesterone for 72 h and washed twice thereafter. GnRH stimulation was carried out with different concentrations (10^{-12}–10^{-6} M) of the decapeptide. Control shows the dose–response curve of LH (left panel) and FSH (right panel) release.

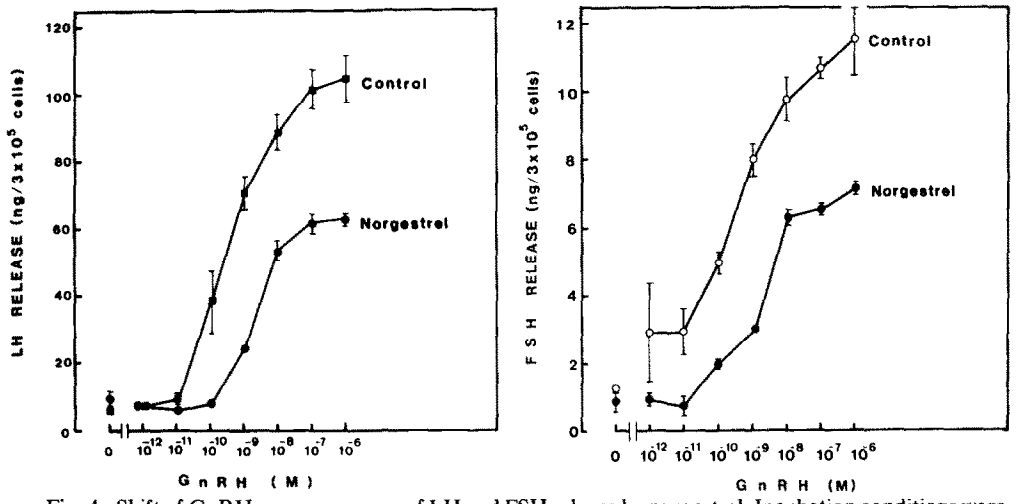

Fig. 4. Shift of GnRH-response curve of LH and FSH release by norgestrel. Incubation conditions were as described in Fig. 3.

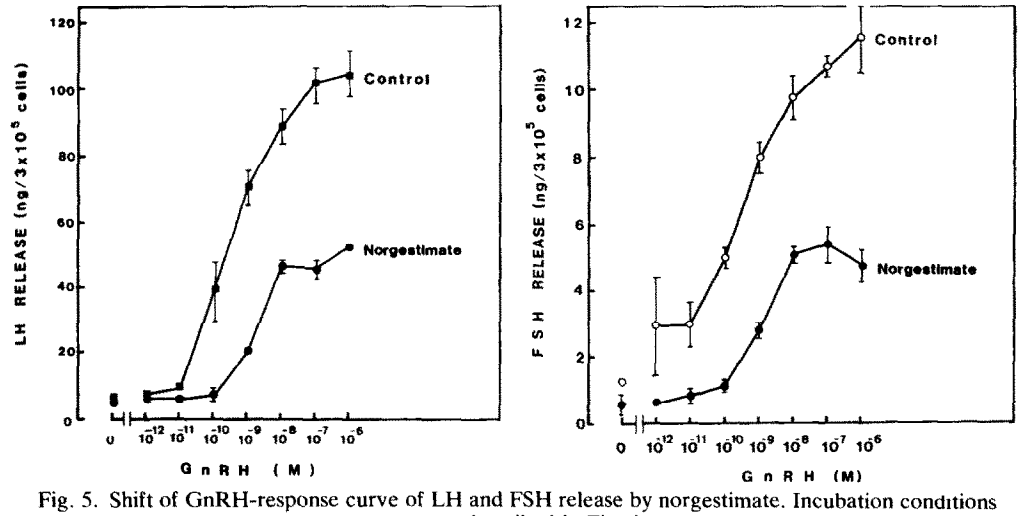

Fig. 5. Shift of GnRH-response curve of LH and FSH release by norgestimate. Incubation conditions were as described in Fig. 3.

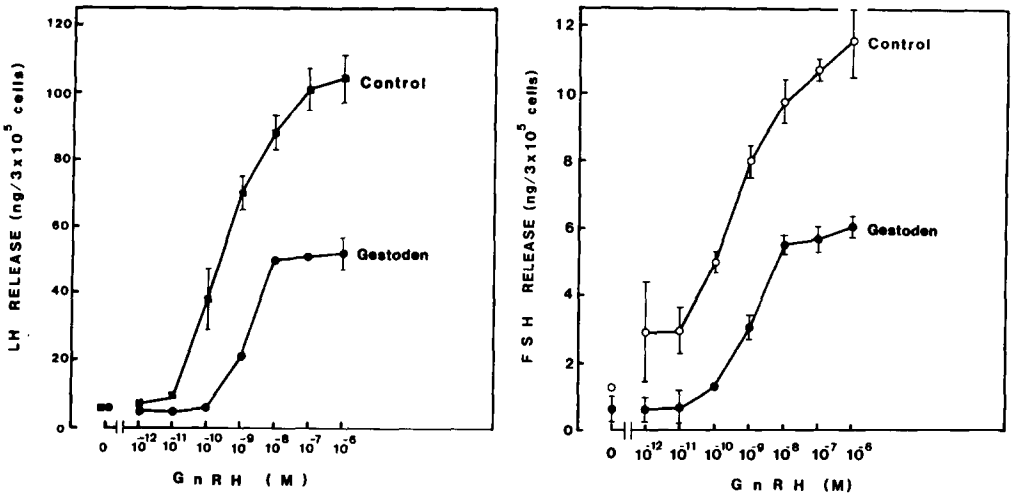

Fig. 6. Shift of GnRH-response curve of LH and FSH release by gestoden. Incubation conditions were as described in Fig. 3.

Inositol phosphate accumulation—a possible site of action of progestins

GnRH stimulates inositol phosphate production following 15-min treatment of cells with the decapeptide (Fig. 7). This hydrolysis of polyphosphoinositides was dose and time dependent in view to GnRH stimulation (not shown here).

Inositol phosphate production was tested in cells preincubated with [^{3}H]inositol with and without GnRH stimulation. Progestins were tested for their effect on the breakdown of polyphosphoinositides by measuring inositol phosphate accumulation in cells pretreated with these steroids. Figure 8 demonstrates an example of gestoden effect on inositol phosphate accumulation. Results are expressed as per cent of control. The difference between GnRH-stimulated and basal values was taken as 100%. Gestoden inhibits the breakdown of polyphosphoinositides to inositol phosphates by 80%, as shown by the reduction of inositol monophosphate (IP_1), inositol

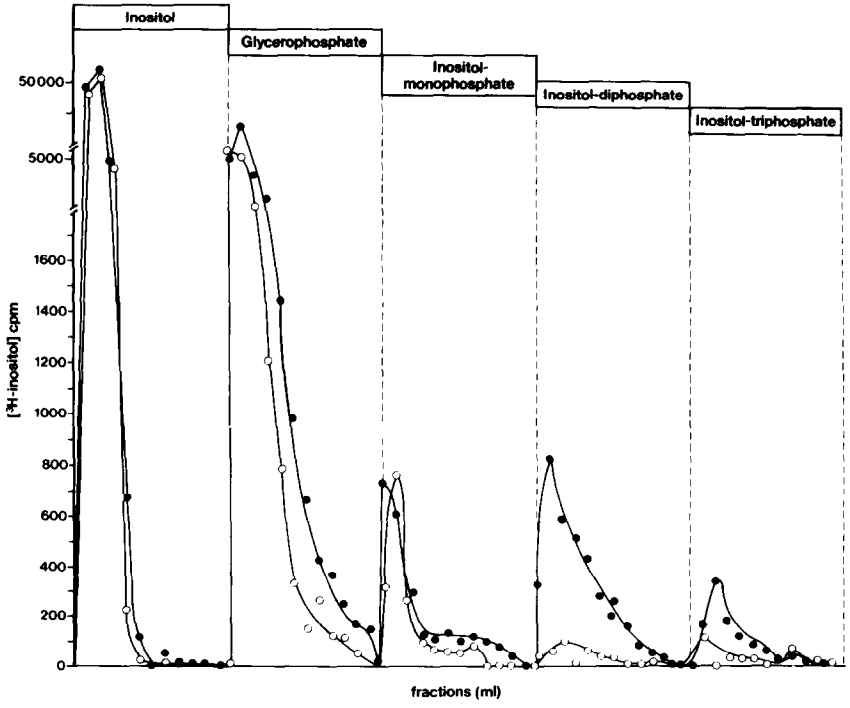

Fig. 7. Separation of inositol phosphates by anion exchange chromatography. Solvents and procedures are explained in Methods. The addition of 10^{-7} M GnRH (●) in comparison to controls (○) results in stimulation of inositol phosphate accumulation.

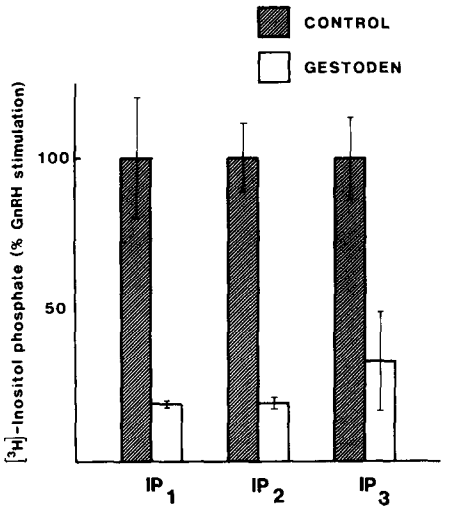

Fig. 8. Inhibition of inositol phosphate accumulation by gestoden. Cells were pretreated with gestoden (10^{-5} M) for 72 h, following two washes. Pituicytes were stimulated with GnRH (10^{-7} M). Control (100%) contained only the solvent (0.01% dimethyl sulfoxide). Inositol monophosphate (IP_1), inositol diphosphate (IP_2) and inositol triphosphate (IP_3) were inhibited by 80% following gestoden pretreatment. Results are expressed as per cent control (mean ±SD, $N = 3$).

diphosphate (IP_2) and inositol triphosphate (IP_3). Inositol phosphates were inhibited by the progestin to the same degree.

DISCUSSION

Oral contraceptives act at different sites to prevent pregnancy in humans. The main effect of these steroid formulations is the inhibition of ovulation by reducing the release of LH and FSH. A site where contraceptives exert most of their gonadotropin inhibitory action has not yet been clearly defined.

Some investigators believe that gonadotropin inhibition by contraceptive steroids occurs mainly in the hypothalamus [25–27], whereas others suggest that these steroids may have a direct suppressive effect on the pituitary gland [2, 28–30]. The administration of GnRH to a large number of women taking combination oral contraceptive steroids for long periods of time resulted in a significantly lower release of both LH and FSH when compared to controls [2, 28–30]. The effect of the same contraceptive steroid formulation upon pituitary gonadotropin function had great individual variations, although GnRH-stimulated LH and FSH release was nearly always consistent for each subject [31]. In all steroid users studied in that publication, the diminished gonadotropin response to GnRH indicated that the contraceptive agents had a direct inhibitory action mainly upon pituitary gonadotropin function [31].

Using an indirect approach, Römmler [32] established a GnRH double stimulation test to further characterize estrogen and progestin effects on the pituitary *in vivo*. This test is performed by the administration of two consecutive GnRH injections of the same dose at an interval of 2 h. The increase of LH and FSH in serum following the first and second injections are measured. In that study [32], various progestins in different doses were compared and a dose-dependent inhibition of pituitary function by progestins was found. Estrogen levels in serum closely correlated with the increase of gonadotropins, which is probably due to the effect of estrogens on gonadotropin synthesis in the pituitary. The gonadotropin increase following the second GnRH injection seems to be a good indicator for the capacity of the gonadotroph for hormone synthesis. In the same study, contraceptive gestagens antagonized the estrogen effect on pituitary LH and FSH release. The anti-estrogenic effect seems to depend on the type and the dose of the progestin.

Daily injections of ethinyl estradiol (50 μg) and norethisterone (1 mg) resulted in an increase of cell response to a GnRH test [33]. During the following days, a suppression of pituitary response was achieved. Further experiments showed that this initial stimulation was probably due to the progestational component [34].

In our *in vitro* experiments, an initial stimulatory effect of norethisterone (4-h pretreatment) could be shown (Fig. 1). Longer preincubations (23–71 h) resulted in an inhibition of GnRH-induced gonadotropin secretion.

The clinical application of progesterone depends mainly on its spectrum of effects. A steroid with strong anti-gonadotrophic and strong anti-estrogenic effects is preferable as a progestational component for a hormonal contraceptive. Recently, several new synthetic progestins have been developed for use in oral contraceptives [35]. In order to distinguish effects of various progestins on gonadotropin release, an *in vitro* bioassay has been developed in this publication.

The influence of gonadal steroids on the time-course of release and synthesis of LH and FSH from rat pituitary glands *in vitro* has been shown earlier [6, 7, 9, 36–44]. This publication deals with various aspects of oral contraceptive progestins on gonadotropin secretion utilizing enzymatically dispersed pituitary cells grown in primary monolayer culture as an *in vitro* bioassay. As shown by others previously [9], progesterone decreases the responsiveness of pituitary cultures to GnRH. Synthetic progestins were more potent than progesterone in reducing gonadotropin release from rat pituitary cells. Several progestins were compared by establishing steroid dose-dependent curves of LH release in cells stimulated by GnRH. In addition, ED_{50} (half-maximal stimulation of LH release by GnRH) curves for individual estrogens have been

analyzed. Progestins reduced the sensitivity of gonadotrophs to release LH and FSH.

Using these different approaches, progestins were characterized according to their inhibitory effect on gonadotropin secretion. Further studies were carried out to specify whether progestins can interact with intracellular mechanisms of gonadotropin-induced LH release. Polyphosphoinositide hydrolysis has been investigated and inositol phosphate accumulation was determined. Progestins were potent inhibitors of inositol phosphate accumulation. All three inositol phosphates (inositol monophosphate, inositol diphosphate, inositol triphosphate) were equally inhibited by progestins. Although phospholipid breakdown is only one possible mediator of GnRH action next to anachidonic acid metabolism and protein kinase C [45–49], it is assumed that the breakdown of inositol phospholipids may also be one of the intracellular sites of progestin action in pituitary cells.

The results from these *in vitro* experiments, however, require cautious interpretation, since these results show only one of several effects of progestins and, in addition, the extrapolation of animal assays to human use needs further studies. Progestins differ greatly in their additional properties and their action usually requires synergism with estrogen, which shows large differences in species. Nevertheless, this *in vitro* bioassay has a potential to serve as a tool to examine one property of progestagens studying antigonadotrophic effects. This approach also allows investigation of some aspects of the molecular sites of progestin action in gonadotrophs.

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