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Effect of porcine follicular fluid preparations on gonadotrophin secretion by the mouse pituitary gland *in vitro*

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SUMMARY

The acute effects of pooled porcine follicular fluid (PFF), before and after various methods of processing to eliminate steroids, were studied on the luteinizing hormone releasing hormone (LH-RH)-induced release of FSH and LH by whole pituitary glands, from 34-day-old mice, incubated *in vitro* for 3–4 h. Charcoal treatment of PFF eliminated the steroids and reduced the inhibitory potency on gonadotrophin secretion. On the other hand, dialysis or ultrafiltration (mol. wt > 10 000) did not reduce the inhibitory activity on gonadotrophin secretion.

Of the three steroids tested, only oestradiol at a concentration of 10^{-10} mol/l inhibited FSH and LH secretion *in vitro*. This inhibitory effect was counteracted by the inclusion of the oestrogen antagonist tamoxifen in the incubation medium. The presence of tamoxifen did not decrease the suppression of FSH and LH induced by PFF, suggesting that the inhibition observed under the conditions of incubation was not due to oestrogen. Preincubation of mouse pituitary tissue for 1 h with PFF reduced the subsequent release of bioactive FSH and LH induced by LH-RH. The inhibitory effect of PFF was rapid and sustained. The continuous presence of PFF throughout the incubation period was not necessary for manifestations of the inhibitory effects on gonadotrophin release. The suppression of gonadotrophin secretion was related to the dose of PFF with the curve showing a biphasic pattern. The degree of FSH suppression was uniformly greater than that of LH, showing the preferential nature of the inhibitory effect of PFF. At high doses of PFF, the degree of FSH suppression was decreased significantly. This effect on LH release was less pronounced.

The inhibition caused by PFF in the in-vitro incubation procedure was not due to destruction of LH-RH or the released gonadotrophins.

INTRODUCTION

There is now good evidence demonstrating that the ovary, like the testis, produces a non-steroidal substance which also participates in the regulation of pituitary gonadotrophin secretion. This has been designated inhibin-F or folliculostatin. Thus, steroid-free follicular fluid from cow (de Jong & Sharpe, 1976), pig (Schwartz & Channing, 1977; Welschen, Hermans, Dullaart & de Jong, 1977), horse (Miller, Wesson & Ginther, 1979), man (Daume, Chari, Hopkinson, Sturm & Hirschhauser, 1978), monkey (Channing, Anderson & Hodgen, 1980) and hamster (Chappel, Acott & Spies, 1979) has been shown to suppress selectively pituitary follicle-stimulating hormone (FSH) secretion *in vivo* and/or *in vitro*. The peptidic nature of the inhibin-like substance in the follicular fluid has been demonstrated (Lorenzen, Channing & Schwartz, 1978; Franchimont, Proyard, Hagelstein, Renard, Demoulin, Bourguignon & Hustin, 1979; de Jong, Smith & van der Molen, 1979) and reports on its partial purification have appeared (Chari, Hopkinson, Daume & Sturm, 1979; Jansen, Steenbergern, de Jong & van der Molen, 1981). Follicular fluid inhibin, which is very potent in suppressing FSH in the female, is apparently less effective in the male (Lorenzen, Dworkin & Schwartz, 1981). In the present study we have examined the acute effect of variously treated porcine follicular fluid preparations on gonadotrophin secretion from male mouse pituitary glands incubated *in vitro* for short periods. As the whole pituitary glands from mice have been shown to respond promptly to synthetic luteinizing hormone releasing hormone (LH-RH) by releasing bioactive FSH and luteinizing hormone (LH) into the medium (Ramasharma, Sairam & Ranganathan, 1981), this served as an appropriate model to examine the inhibitory effects of follicular fluid on gonadotrophin secretion.

MATERIALS AND METHODS

Preparation of porcine follicular fluid (PFF)

Pooled PFF samples were sent to us on dry-ice by the Contraceptive Development Branch of the NICHHD, Bethesda, Maryland, U.S.A. They had been collected by aspiration of pig follicles removed shortly after slaughter and were pooled in batches of 500 ml. The data included in the present studies are derived from pooled follicular fluid batch labelled SPO-44. After arrival in our laboratory, this was kept frozen at −70 °C until use. The following preliminary treatments were performed at 4 °C, resulting in different preparations of the follicular fluid material. When they were not lyophilized, the different fractions were stored in small samples at −20 °C or −70 °C after restoring the original volume. Unless indicated otherwise in this paper, PFF refers to untreated follicular fluid.

Untreated PFF

The frozen PFF was thawed in a stream of cold air and centrifuged at 9800 *g* for 1 h in a Beckman J-21 B centrifuge to remove cellular debris. The supernatant fraction was carefully separated and used as untreated PFF.

Charcoal-treated PFF (CT-PFF)

Untreated PFF (50 ml) was stirred with charcoal (30 mg/ml; Norit-A; Fisher, New Jersey, U.S.A.) for 3 h and centrifuged for 30 min at 12 100 *g*. The supernatant fraction was filtered twice through Whatman No. 1 filter paper to remove all particulate matter.

Dialysed PFF

Untreated PFF was dialysed against distilled water using Spectrapor 3 tubing (molecular weight cut-off 3500; Spectrum Medical Industries, California, U.S.A.). In some experiments, the PFF was also dialysed using other membranes (cut-off 6000–8000 or 12 000–14 000).

Porcine follicular fluid retentate (PFF-R) and ultrafiltrate (UF-PFF)

Untreated PFF (50 ml) was subjected to ultrafiltration using a PM-10 membrane (molecular weight cut-off 10 000; Amicon, Massachusetts, U.S.A.). The retentate and ultrafiltrate were recovered by lyophilization and kept at 4 °C. When required, appropriate amounts equivalent to the original volume of PFF were weighed and reconstituted in the incubation medium.

Animals

Male Swiss albino mice of the Hauska strain were obtained from Charles River Canada, Montreal, Canada, when they were 30 days of age. They were maintained until day 33–34 under standard laboratory conditions with food and water available *ad libitum*. As pointed out earlier (Ramasharma *et al.* 1981), it was important to obtain animals of the same age and uniform body weight (18–20 g on day 33–34) in order to obtain consistent results.

In-vitro incubation procedure

The details of the incubation procedure for the whole pituitary glands from 34-day-old mice have been reported recently (Ramasharma *et al.* 1981). The mice were killed under light anaesthesia. The pituitary glands were carefully removed and placed in a flask containing Dulbecco's modified Eagle's medium oxygenated with 95% O_2 : 5% CO_2, pH 7·4 with 1 mg bovine serum albumin/ml (Sigma, St Louis, Missouri, U.S.A.) (DMEM–BSA). After collection of 50–60 pituitary glands within about 1 h, the pool of tissues was rinsed at 4 °C with fresh medium and preincubated for 30 min at 37 °C in a metabolic shaker under an atmosphere of 95% O_2 : 5% CO_2. After preincubation, the pituitary glands were distributed (one per tube) to 12 × 75 mm disposable glass tubes and incubated with various PFF preparations in 500 µl DMEM–BSA for 60 min. At the end of this period, 3 ng synthetic LH-RH (Ayerst Laboratories, Montreal, Canada) in 500 µl DMEM–BSA were delivered into each tube. The incubation was then continued for a further 3 h. This dose of LH-RH was earlier shown to result in maximal release of both FSH and LH in 3 h (Ramasharma *et al.* 1981) and is within the range used in most incubation studies using dispersed pituitary cells. At the end of the fourth hour the medium was separated from the pituitary tissue by decantation and both were stored at −20 °C until assayed for gonadotrophins. In some instances the gonadotrophins were assayed immediately. For the assay of gonadotrophins in the pituitary glands the tissue was homogenized in an all-glass tissue grinder with 1 ml 0·01 M-phosphate buffer, pH 7·2 and centrifuged for 10–15 min at 2900 *g*. Suitable samples of the supernatant fractions were used for the assay.

Analysis of the gonadotrophins

Follicle-stimulating hormone and LH in the medium and pituitary extract were determined by the specific radioreceptor assays as employed in our previous studies (Ramasharma *et al.* 1981). ^{125}I-Labelled ovine FSH and bull testicular membrane preparation as receptor were used for the FSH assay. The LH assay utilized ^{125}I-labelled ovine LH and an homogenate of ovaries from pseudopregnant rats (Lee & Ryan, 1975). Both ovine FSH (S1552BR) and ovine LH (S1322AR), used for iodination as well as standards, were highly purified samples prepared in our laboratory. The biological potency of the ovine FSH preparation was 100 × NIH-FSH-S10 and that of ovine LH was 2–3 × NIH-LH-S19. The different concentrations of PFF preparations and steroids used in the present experiments had no effect on the FSH and LH receptor assays (data not shown).

Since the FSH and LH to be estimated were contained in the DMEM–BSA from the incubations, the standard curves for FSH and LH were run in the presence of an equivalent concentration of the same medium to compensate for any non-specific effects. The assays have been validated for the mouse gonadotrophins in the tissue extract and the medium (Ramasharma *et al.* 1981). The concentrations of gonadotrophins were computed on a logit-log basis using a Hewlett-Packard desk-top calculator.

Analysis of steroids

Steroids in the preparations of PFF were extracted by diethyl ether and estimated by radioimmunoassay using specific antibodies supplied by Immunotech, Montreal, Canada. The detectable range was 25–500 pg/tube for oestradiol, 50–1000 pg/tube for testosterone and 10–1000 pg/tube for progesterone.

Statistical analysis

All results were expressed as means ± S.E.M. and analysed for significance by Duncan's multiple range test or Student's *t*-test where necessary. Differences were considered to be significant at $P \leqslant 0·05$.

RESULTS

Steroid content in PFF preparations

Although a number of steroids are present in the follicular fluid, we chose to determine only three, which may be expected to modulate the release of gonadotrophins. The concentrations of oestradiol, testosterone and progesterone are shown in Table 1. After charcoal treatment almost 99% of the steroids were removed, confirming many previous observations. Dialysis of the fluid reduced the steroid content by about 50–60% but ultrafiltration was uniformly more effective. The additional removal in the latter procedure is attributable to adsorption of the steroids by the membrane (according to the manufacturer).

Table 1. *Concentration of oestradiol, testosterone and progesterone in porcine follicular fluid (PFF). Oestradiol, testosterone and progesterone in PFF were extracted with diethyl ether and measured by radioimmunoassay. Details of treatment of PFF are described in Materials and Methods*

Treatment of PFF	Oestradiol (10^{-10} mol/l)	Testosterone (10^{-10} mol/l)	Progesterone (10^{-10} mol/l)
Untreated	304·5	1514·9	2277·0
Charcoal-treated	3·0	5·3	6·0
Dialysed	159·3	664·3	1098·7
Retentate	112·0	468·6	372·6
Ultrafiltrate	77·0	61·8	112·1

Effect of treated PFF on LH-RH-induced gonadotrophin secretion

Untreated PFF and CT-PFF (Fig. 1)

Preincubation of the mouse pituitary glands with different amounts (0·08–10 μl) of PFF before the addition of LH-RH resulted in a progressive decrease in the accumulation of gonadotrophin in the medium. As the incubation volume was 1 ml, the addition of untreated PFF ranged from 0·008 to 1% by volume.

Charcoal-treated PFF gave a different result with respect to patterns of inhibition. Follicle-stimulating hormone secretion was suppressed at the intermediate doses of 0·4 and 2 μl per tube. A similar tendency was also seen with LH, but in this case the 10 μl dose was also inhibitory. After charcoal treatment the tendency for dose-dependent suppression of both FSH and LH appeared to have been lost in comparison to the respective doses of untreated PFF.

Dialysed PFF (Fig. 2)

Dialysed PFF, irrespective of the type of membrane used (see Materials and Methods), resulted in a decreased suppression of both FSH and LH. The differences in the inhibition before and after dialysis were not statistically significant.

Porcine follicular fluid retentate and UF-PFF (Fig. 3)

In the in-vitro incubations, PFF-R (mol. wt > 10 000 daltons) suppressed FSH secretion to a degree slightly less than that induced by untreated PFF, suggesting some decrease of inhibitory activity ($P<0.05$ at 2 and 0·4 μl doses for FSH). Ultrafiltered PFF suppressed FSH secretion only at the highest dose tested (10 μl equivalent) and this was much less pronounced than either untreated PFF or PFF-R, indicating retention of most of the inhibin activity in the latter fraction. The effects of PFF-R and UF-PFF on LH secretion were distinctly different. The suppression of LH by PFF-R was almost the same as that induced by untreated PFF and no significant difference was seen between 2 and 0·4 μl.

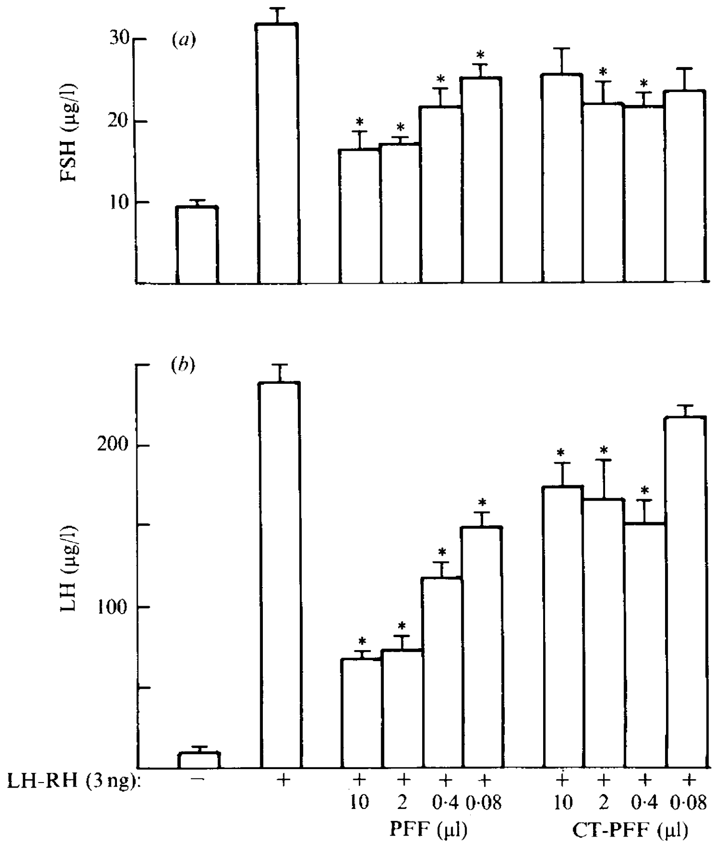

Fig. 1. Effect of untreated porcine follicular fluid (PFF) on (*a*) FSH and (*b*) LH secretion. Pituitary glands were removed from 34-day-old male mice and preincubated for 30 min. After distribution to each tube untreated PFF or charcoal-treated PFF (CT-PFF) was added in 500 μl medium and incubated for 1 h at 37 °C. This was followed by the addition of 500 μl medium which contained 3 ng synthetic luteinizing hormone releasing hormone (LH-RH). The incubation was continued for 3 h, at the end of which the pituitary glands and medium were separated by decantation. Gonadotrophins in the medium were measured by radioreceptor assay using highly purified ovine FSH (S1552BR) and LH (S1322AR) prepared in our laboratory. Values are means ± S.E.M. ($n = 5$). $*P < 0·05$ compared with LH-RH (Student's *t*-test). Differences between untreated PFF and CT-PFF for FSH levels at respective doses were not statistically significant by Student's *t*-test. By Duncan's multiple range test, the FSH and LH levels among the groups treated with varying amounts of untreated PFF respectively were statistically significant.

Ultrafiltered PFF had no effect on LH secretion at any dose. On the contrary, the amount of LH accumulating in the medium appeared to increase in the presence of all doses (0·4–10 μl equivalent) of UF-PFF.

Effect of steroids on gonadotrophin secretion by the mouse pituitary gland

As indicated in Table 1, it is clear that untreated PFF contained gonadal steroids. Since we observed potent inhibition of gonadotrophin secretion in the presence of untreated PFF, it was of interest to examine whether the addition of steroids alone had any effect in the mouse system. Accordingly, the three steroids calculated to be in amounts equivalent to that found in 2 and 10 μl of untreated PFF were added to each tube, i.e. oestradiol at $5·8 \times 10^{-11}$ or $3·0 \times 10^{-10}$ mol/l, testosterone at $3·0 \times 10^{-10}$ or $1·5 \times 10^{-9}$ mol/l and

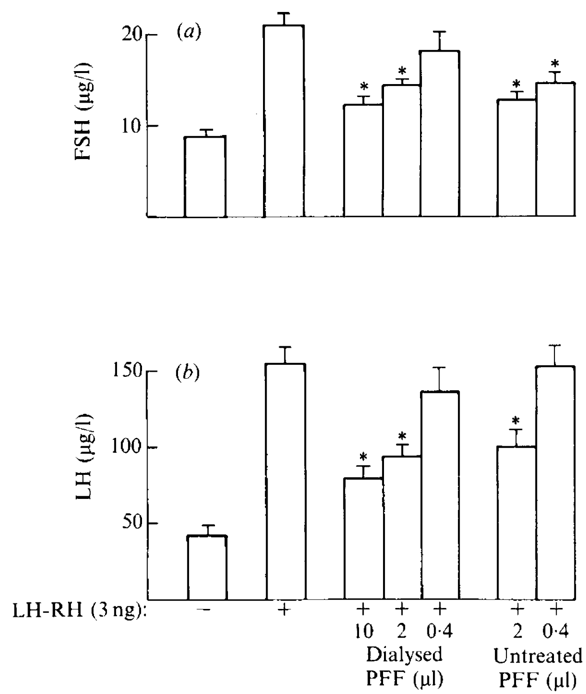

Fig. 2. Effect of dialysed porcine follicular fluid (PFF) on (*a*) FSH and (*b*) LH secretion. The procedure of the incubation was the same as in Fig. 1. Dialysed PFF or untreated PFF in 500 μl medium was added during the first hour of incubation. Values are means ± S.E.M. ($n = 5$). $^*P < 0·05$ compared with luteinizing hormone releasing hormone (LH-RH) (Student's *t*-test).

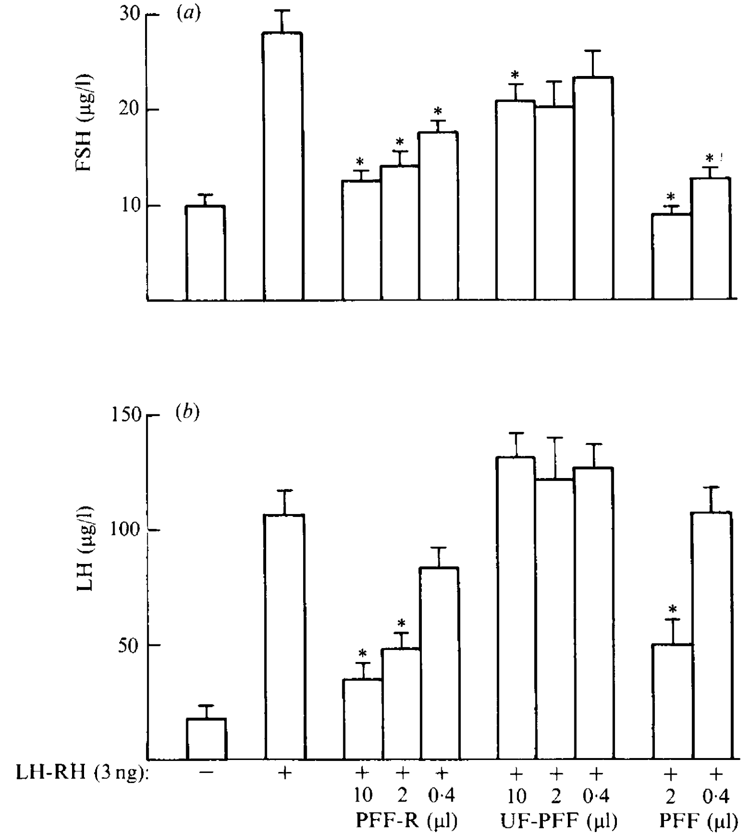

Fig. 3. Effect of porcine follicular fluid retentate (PFF-R) and ultrafiltrate (UF-PFF) on (*a*) FSH and (*b*) LH secretion. Follicular fluid retentate or UF-PFF in 500 μl medium was added during the first hour of incubation (see legend to Fig. 1). The amounts of PFF-R and UF-PFF are equivalents of their original volume. Values are means ± S.E.M. ($n = 5$). $^*P < 0·05$ compared with luteinizing hormone releasing hormone (LH-RH) (Student's *t*-test). The differences in the groups treated with PFF-R were statistically significant by Duncan's multiple range test.

progesterone at $4{\cdot}5\times10^{-10}$ or $2{\cdot}2\times10^{-9}$ mol/l. The results shown in Fig. 4 were obtained from these experiments. Testosterone and progesterone alone had no effect on either FSH or LH secretion induced by LH-RH. In three of four experiments oestradiol alone reduced both FSH and LH secretion. Combination of all three steroids also gave a similar result. Oestradiol at concentrations less than $5{\cdot}8\times10^{-11}$ mol/l had no inhibitory effect on the release of the gonadotrophins.

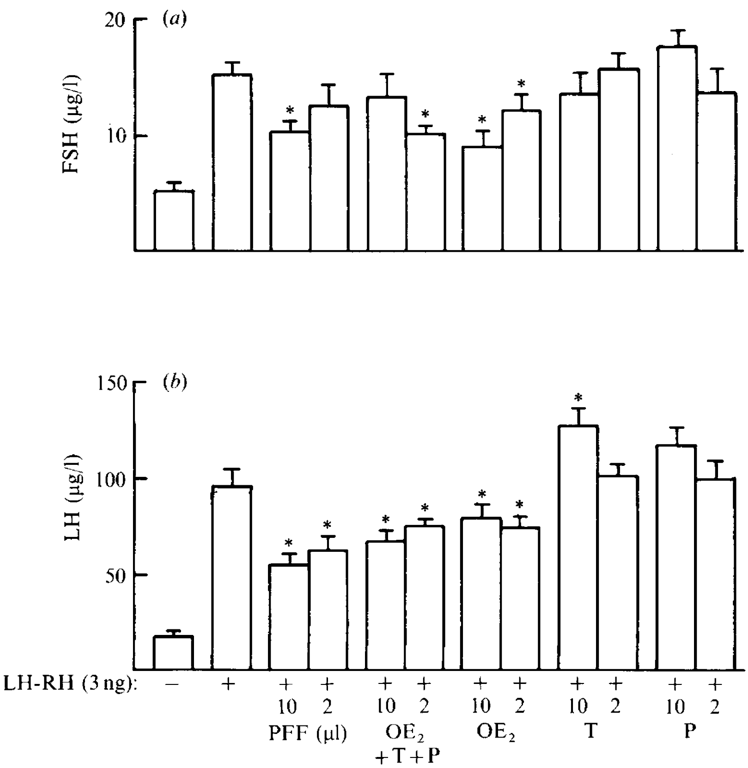

Fig. 4. Effect of steroids on (*a*) FSH and (*b*) LH secretion. Oestradiol (OE_2), testosterone (T) and progesterone (P) were added independently or together in 500 µl medium during the first hour of incubation (see Fig. 1). The amounts of steroid tested were $5{\cdot}8\times10^{-11}$ and $3{\cdot}0\times10^{-10}$ M-OE_2, $3{\cdot}0\times10^{-10}$ and $1{\cdot}5\times10^{-9}$ M-T and $4{\cdot}5\times10^{-10}$ and $2{\cdot}2\times10^{-9}$ M-P, which correspond to their respective concentrations calculated to be in 2 or 10 µl of untreated porcine follicular fluid (PFF) (Table 1). Values are means ± S.E.M. (n = 5). *$P<0{\cdot}05$ compared with luteinizing hormone releasing hormone (LH-RH) (Student's *t*-test).

To rule out the contribution of oestradiol in PFF under our experimental conditions, the experiments were repeated in the presence of the well defined oestrogen antagonist, tamoxifen (tamoxifen citrate, Tmx; ICI Americas Inc., Wilmington, Delaware, U.S.A.). Tamoxifen has been shown to reverse the inhibitory effects of oestrogen on FSH secretion and the stimulatory effects on LH secretion in sheep pituitary cells (Miller & Huang, 1981). In the mouse pituitary incubation system the concurrent addition of tamoxifen (10^{-6} mol/l) eliminated the inhibitory effect of oestradiol on both FSH and LH secretion (Fig. 5). However, the suppression of both gonadotrophins induced by untreated PFF was not influenced by the inclusion of tamoxifen alone in the incubation medium. Thus, these results confirm that the suppression observed in the mouse incubation system (Figs 1, 2, 3 and 6) was not due to oestradiol. We have reported similar data on the failure of tamoxifen

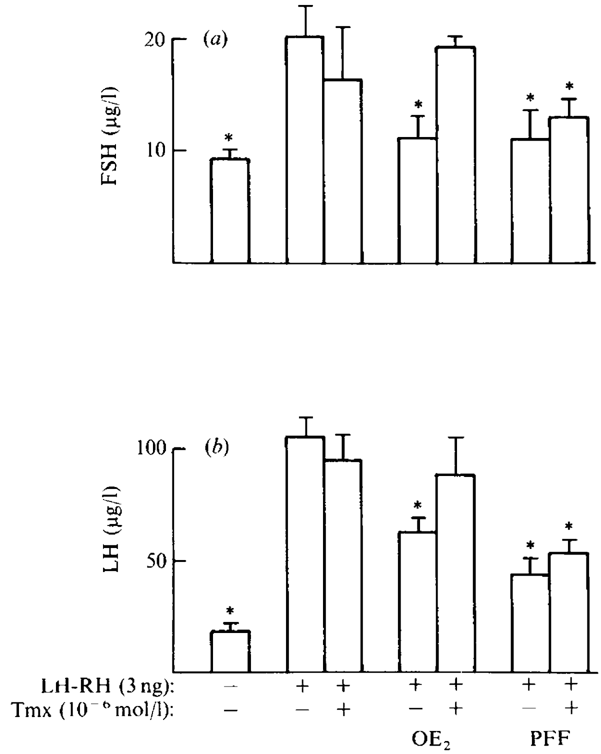

Fig. 5. Effect of the oestrogen antagonist, tamoxifen (Tmx), on (*a*) FSH and (*b*) LH secretion by the mouse pituitary gland. Tamoxifen (10^{-6} mol/l) was added to the incubation medium (see Fig. 1) with 10 μl untreated porcine follicular fluid (PFF) or $3{\cdot}0 \times 10^{-10}$ M-oestradiol (OE_2), which corresponds to the amount found in 10 μl untreated PFF. Values are means ± S.E.M. ($n = 5$). *$P < 0{\cdot}05$ compared with luteinizing hormone releasing hormone (LH-RH) (Student's *t*-test).

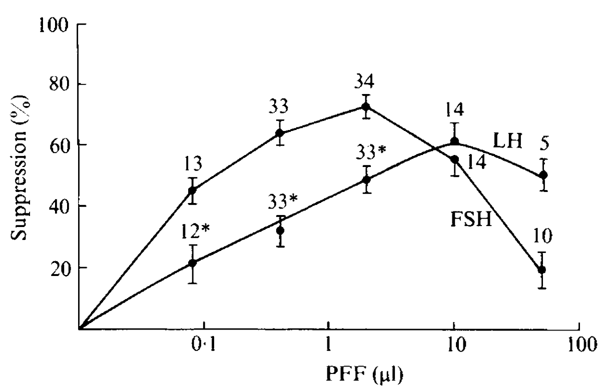

Fig. 6. Effect of untreated porcine follicular fluid (PFF) on FSH and LH secretion. The percentage suppression of gonadotrophin secretion by the different amounts of untreated PFF in seven experiments is shown. The procedure of the incubation was described in the legend to Fig. 1. Values are means ± S.E.M. *$P < 0{\cdot}05$; the degree of LH suppression is significantly different from that observed for FSH at the respective doses (Student's *t*-test). The numbers of treatments considered in the evaluation are shown above each point. By Duncan's multiple range test there was significant difference in the degree of suppression noted in the presence of PFF for both FSH and LH.

to antagonize the inhibitory actions of PFF on secretion of FSH from sheep pituitary cells (Sairam, Kato, Manjunath, Ramasharma, Miller, Huang & MadhwaRaj, 1982).

The effect of pre-exposure of mouse pituitary glands to PFF on gonadotrophin secretion and the effect of PFF on the secretion induced by LH-RH

In all previous experiments (Figs 1–5) PFF was added in the first hour followed by co-incubation with added LH-RH during the next 3 h. Such conditions lead to two possible interpretations of the observed reduction in gonadotrophin secretion, one possibility being that the added PFF in some manner inactivates the LH-RH or secondly that the PFF destroys gonadotrophins. Two experiments were designed to eliminate these two possibilities. In the first set, 500 µl DMEM–BSA containing PFF was added for the first hour only. The medium was then removed and the pituitary tissue in each tube was rinsed twice with fresh medium. Thus, each tissue was in contact with the PFF for only 1 h. This was then followed by the addition of 1 ml DMEM–BSA containing 3 ng LH-RH and incubation was continued for 3 h. Despite the removal of PFF after the first hour, both FSH and LH released into the medium were significantly inhibited. In another similar experiment, 500 µl DMEM–BSA containing 3 ng LH-RH were first added to the pituitary glands. Untreated PFF in 500 µl medium was added at the same time as LH-RH or later (15 min or 1 h). After incubation for 3 h FSH and LH were assayed. Irrespective of whether the PFF was added at the same time as LH-RH or shortly thereafter, there was no decrease in the release of either gonadotrophin into the medium. It became evident from these results, that untreated PFF did not contain substances that inactivated LH-RH or destroyed gonadotrophins under our experimental conditions.

Cumulative data on the effects of PFF on FSH and LH secretion

The data from seven experiments were pooled and compared with regard to the efficacy of untreated PFF to influence FSH and LH secretion induced by LH-RH in the mouse pituitary gland. As noted in Fig. 6, untreated PFF showed a dose-dependent suppression between 0·08 and 2 µl. The maximum suppression in most instances ($72{\cdot}9 \pm 3{\cdot}8\%$) was seen at the 2 µl dose. At the higher doses of 10 and 50 µl the suppression was decreased (i.e. the amount of FSH released into the medium was not significantly different from the LH-RH-treated group; note the 50 µl values in Fig. 6). As with FSH, LH release was also suppressed in a dose-dependent manner from 0·08 to 10 µl. In the presence of 50 µl PFF, the suppression was less than that at 10 µl but the difference was not statistically significant. Comparison of the degree of FSH and LH suppression at the 0·08, 0·4 and 2 µl concentrations revealed statistically significant differences ($P < 0{\cdot}005$), indicating that FSH was suppressed to a greater extent than LH at equivalent concentrations. Data in Fig. 6 show that about 0·2 and 2 µl of PFF were necessary to bring about a 50% decrease of secretion of FSH and LH respectively. Thus from our data the sensitivity for FSH suppression appears to be tenfold greater than that for LH. This is also in agreement with data reported in the literature.

In these short-term incubations, no significant effects of the PFF fractions on the pituitary content of the gonadotrophins were detectable (data not shown).

DISCUSSION

The aim of the present set of experiments was to find out the characteristics of inhibition produced by the acute action of PFF on mouse pituitary glands incubated *in vitro*. Untreated PFF suppressed the LH-RH-induced release of both gonadotrophins in a dose-dependent manner (Figs 1 and 6) and there was a clear preferential effect on FSH secretion. The incubation was too short (1–4 h) in the present study to observe any effect of PFF on basal release of FSH and LH (data not shown). No decrease of either gonadotrophin was seen in 4 h without LH-RH. The results with PFF, PFF-R and dialysed PFF (Figs 1–3) are in good agreement with published data (Schwartz & Channing, 1977; Franchimont *et al.* 1979; Jansen *et al.* 1981; Ramasharma *et al.* 1981), indicating a molecular weight >10 000 daltons, and validate our test system. Data from the use of treated PFF as well as the oestrogen antagonist, tamoxifen (Figs 1–5), support the view that the inhibition observed in the in-vitro study is not due to a steroid component, a conclusion which is also supported by many studies in the literature (e.g. Schwartz & Channing, 1977; Welschen *et al.* 1977; Lorenzen *et al.* 1978, 1981).

The use of whole pituitary glands from mice in the incubation system of short duration did not pose any particular problem in eliciting a prompt response to added synthetic LH-RH, confirming our recent study (Ramasharma *et al.* 1981). This also suggests that penetration of the LH-RH or PFF into the secreting cells, at least at the periphery of the anterior portion in the whole pituitary gland, must have occurred to a reasonable extent. The argument that this could be a source of variation after LH-RH response is not supported by our data. The degree of variation in LH-RH response in the incubation of the whole pituitary gland, as calculated by the degree of stimulation in each experiment (i.e. LH-RH stimulated/control) was of approximately the same order as with dispersed pituitary cell cultures (Ying, Ling, Böhlen & Guillemin, 1981) which are more time consuming. Since each experiment always included appropriate sets of controls, comparisons made within the experiment were valid in our study.

Steroids are well known to have various effects on gonadotrophin secretion *in vivo* (Barraclough, Wise, Turgeon, Shander, DePaolo & Rance, 1979) and *in vitro* on dispersed cells (Drouin & Labrie, 1981; Miller & Wu, 1981) and hemipituitary glands (de Koning, Van Dieten & Van Rees, 1976). In dispersed anterior pituitary cell cultures from rats, oestradiol (10^{-9} mol/l) increased basal as well as LH-RH-stimulated LH secretion but not that of FSH (Drouin & Labrie, 1981). Progesterone in the same study was reported to increase basal and LH-RH-induced FSH but not LH secretion. In rat hemipituitary glands LH was suppressed in 3 h by including oestradiol at 15 ng/ml ($5{\cdot}5 \times 10^{-8}$ mol/l) (de Koning *et al.* 1976) but O'Conner, Allen & Mahesh (1979), working with adult rat tissue, observed no effect at a wide range (10–50 000 pg/ml) of steroid concentrations. The response of the immature mouse pituitary gland to LH-RH was affected by the presence of oestradiol at 10^{-10} mol/l (Fig. 4) but not by testosterone or progesterone.

The rapid effect of PFF, resulting in the inhibition of LH-RH-induced gonadotrophin release, was striking in the present study. Preincubation for 1 h was adequate to observe significant inhibition of gonadotrophin secretion. Although the constant presence of PFF together with LH-RH was not required to inhibit gonadotrophin release, the incubation may be continued without removing the PFF, as a matter of convenience. Although the maximum suppression obtained under the present protocol was about 72% for FSH, preincubation of the pituitary tissue for a longer period might lead to a more complete blockade of LH-RH response. In in-vivo test systems (Lorenzen *et al.* 1978) or dispersed pituitary cells *in vitro* (de Jong *et al.* 1979) several hours or days are required for observing effective suppression of FSH secretion. It is unlikely that the inhibition we have noted could have been due to either a destruction of the LH-RH and/or the released gonadotrophins. In other experiments we have included the PFF at concentrations of 100 µl per tube (data not shown) in our FSH and LH radioreceptor assays and observed no decrease in the binding of the labelled hormone to the receptor, excluding any destruction of the labelled hormone or the receptor.

Although charcoal treatment of PFF is highly effective in removing steroids, we also noted losses of inhibin activity in CT-PFF (Fig. 1). A similar loss of activity by charcoal treatment has been noted in the purification of inhibin from rete testes fluid (Baker, Eddie, Higginson, Hudson & Niall, 1982). It is very likely that follicular fluid inhibin, which is reported to be a hydrophobic protein (Jansen *et al.* 1981), could be adsorbed on to the activated charcoal and thus be partly removed by such treatment.

The paradoxical effects of higher concentrations of PFF deserve some comment. While the paradoxical decrease of inhibition of FSH release was more clear-cut at higher doses of PFF (Fig. 6), there was also a similar tendency for LH. As noted above, the presence of gonadotrophin in the added PFF cannot account for these effects. It is possible that the presence of LH-RH-like substances such as gonadocrinin (Ying *et al.* 1981) could be responsible for the observed decrease of inhibition of FSH release. When higher doses of PFF are used, the ratio of inhibin : gonadocrinin may favour the action of the latter, resulting in decreased inhibition. As ovarian gonadocrinin is apparently of a molecular weight less than 3500 daltons, it may be expected to be effectively removed (Ying *et al.* 1981) by dialysis. However, dialysed PFF or other partially purified preparations also showed the paradoxical effects of decreased inhibition at high doses. Thus it is felt that the apparent presence of gonadocrinins cannot fully account for the paradoxical effects of higher doses of PFF and its fractions. Further studies are required to analyse these effects in detail.

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