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INHIBITION OF GONADOTROPIN SENSITIVE ADENYLATE CYCLASE BY OVARIAN FOLLICULAR FLUID

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SUMMARY

Follicular fluid obtained from medium or large bovine ovarian follicles inhibited ovarian luteinizing hormone/human chorionic gonadotropin sensitive adenylate cyclase in a dose-dependent manner (I_{50}=3 mg follicular fluid protein/ml). The inhibitory activity was excluded by Sephadex G-10 and was fully retained following treatment with charcoal. Fluoride-stimulated enzyme activity was not inhibited. Binding of ^{125}I human chorionic gonadotropin to ovarian plasma membranes was only slightly reduced by the follicular fluid. The post-microsomal supernatant of homogenates from ovaries of immature (27-day-old) rats collected 24-36 h after treatment with 15 i.u. of pregnant mare serum gonadotropin also inhibited luteinizing hormone-sensitive adenylate cyclase. The extent of this inhibition seemed to decline with follicular maturation. The possibility is raised that ovarian sulfated glycosaminoglycans are responsible for the observed inhibition of adenylate cyclase.

INTRODUCTION

Ovarian follicle maturation is accompanied by antrum formation. It has been suggested that the antral fluid plays a role in the ovulatory rupture of the follicle. This may occur by elevation of the intrafollicular osmotic pressure following depolymerization of macromolecular constituents of this fluid (1,2). More recently, follicular fluid has been implicated in the inhibition of ovum maturation (3) and of granulosa cell luteinization *in vitro* as judged by morphological transformation, progesterone secretion and accumulation of cyclic AMP in response to luteinizing hormone (LH)(4). Furthermore, follicular fluid was shown to contain heparin-like substances (5-7), and heparin was shown to inhibit LH-sensitive adenylate cyclase in ovarian plasma membrane preparations (8-10). Here we present evidence for the inhibition of LH-sensitive adenylate cyclase by bovine follicular fluid and by a soluble

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fraction obtained from rat ovarian homogenates. The ability of this fraction to inhibit ovarian adenylate cyclase was significantly reduced concomitant with completion of follicular maturation.

MATERIALS AND METHODS

Preparation of purified ovarian plasma membranes, determinations of adenylate cyclase activity, binding of ^{125}I-human chorionic gonadotropin (^{125}I-hCG) and protein were as described elsewhere (11).

Bovine follicular fluid was collected from medium to large ovarian follicles (1.0 - 1.5 cm in diameter) of cycling Holstein cows at slaughter. The fluid was placed in ice. Cell debris were removed by centrifugation at 2000xg for 30 min.

26-day-old rats were injected subcutaneously with 15 i.u. of pregnant mare serum gonadotropin (PMSG) at 1 p.m. Animals (8 per group) were sacrificed 24 h, 36 h, 42 h or 48 h later, and ovaries were collected in ice-cold phosphate-buffered saline. The glands were cut into small pieces and homogenized in distilled water (1:4 w/v) with an all-glass motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10^5xg for 1 h at 4° and the supernatant was collected. The fractions were stored at -20°C. Ovaries obtained from PMSG-treated rats were processed for histological examination as described elsewhere (12). Sections stained with 1% methylene-blue were photographed using photomicroscope III (Zeiss).

RESULTS

Gonadotropin-sensitive adenylate cyclase in rat ovarian plasma membranes was inhibited by bovine follicular fluid. Inhibition was dose-dependent (Fig. 1A). Fifty percent inhibition (I_{50}) of LH-hCG stimulated adenylate cyclase activity was seen at a concentration of 3 mg of follicular fluid protein/ml. This concentration corresponds to a 1:30 dilution of the natural fluid obtained. By contrast, fluoride-sensitive enzyme showed only little or no inhibition, even at a concentration of 11 mg/ml.

Bovine follicular fluid inhibited ^{125}I-hCG binding to purified ovarian plasma membranes by only 18% at 3 mg protein/ml of the fluid, and by 37% at 11 mg/ml (Fig. 1B). The inhibitory activity of bovine follicular fluid was fully recovered from the effluent of a Sephadex G-10 column in the void volume, suggesting a molecular weight in excess of 700 daltons. Treatment of follicu-

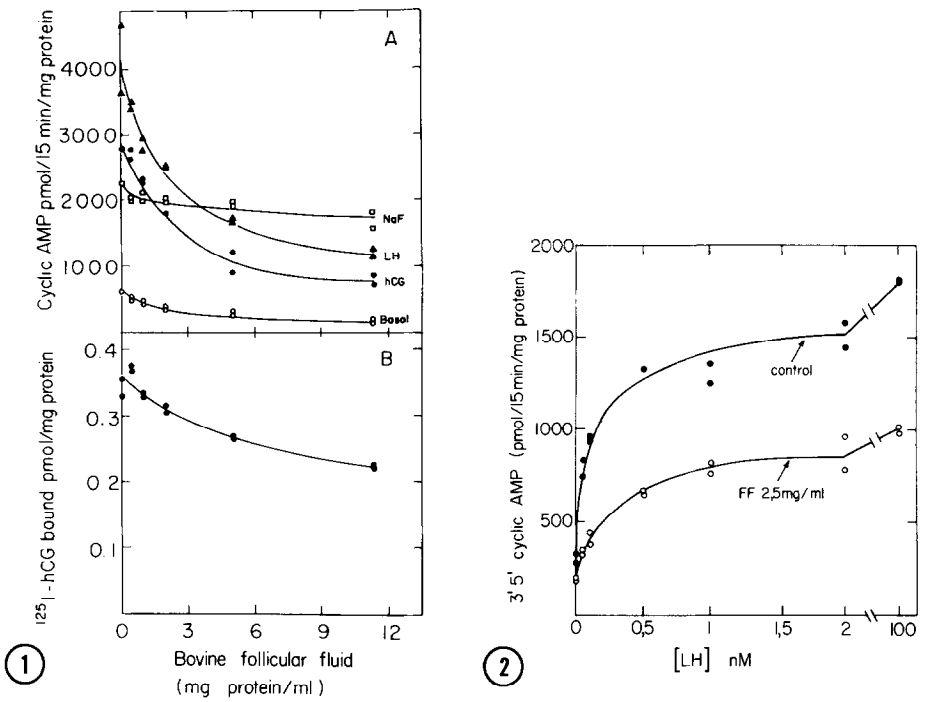

Figure 1. Inhibition of LH/hCG-sensitive adenylate cyclase and hCG binding by bovine follicular fluid.
1A. Adenylate cyclase activity unstimulated (Basal) or stimulated by NaF (10 mM), LH (10^{-7}M) or hCG (10^{-8}M) was determined in the presence of increasing concentrations of bovine follicular fluid. Each assay contained 3 µg of membrane protein. All other details were as described in the Methods section.
1B. ^{125}I-hCG binding was determined at increasing concentrations of bovine follicular fluid. Each assay system contained 10 µg of ovarian plasma membranes. ^{125}I-hCG concentration was 1.7 nM.

Figure 2. The effect of bovine follicular fluid on the activity of LH - sensitive adenylate cyclase at various LH concentrations. Adenylate cyclase activity was determined at the indicated concentrations of LH in the absence (•) or in the presence (o) of bovine follicular fluid (2.5 mg protein/ml) in the reaction mixture. Each assay system contained 3 µg of plasma membrane protein. All other details were described in the Methods section.

lar fluid with charcoal to remove steroids (4) and nucleotides did not alter the inhibitory activity of follicular fluid.

The inhibition of follicular fluid of LH-sensitive adenylate cyclase was found to be independent of LH concentrations. Bovine follicular fluid (2.5 mg protein/ml) inhibited about 50% of the adenylate cyclase activity stimulated by LH in the concentration range 0.1 - 100 nM (Fig. 2).

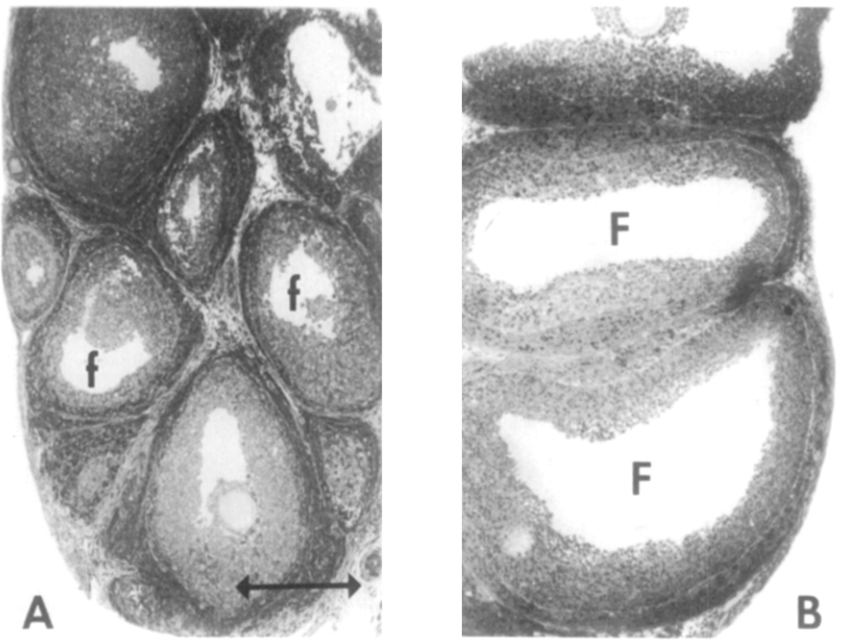

Figure 3. Histological examination of rat ovaries following treatment with PMSG.
Sections of ovaries of 26-day-old rats after treatment with 15 i.u. of PMSG for 24 h (A) or 48 h (B) are presented. Note small and medium size follicles with small antrum (f) occupying the ovarian tissue in A. Large antral follicles (F) are seen in section B. Calibration = 300 μM.

In order to examine whether the inhibition of ovarian adenylate cyclase is affected by follicular maturation, a post-microsomal fraction of homogenates obtained from ovaries at various stages of development was tested. PMSG treatment at the dose-level chosen causes synchronized growth of a large group of follicles: 24 h after injection, many small and medium-sized antral follicles were observed (Fig. 3a), whereas after 48 h most of the ovarian volume was occupied by large antral follicles (Fig. 3b).

Incubation of rat ovarian plasma membranes with the 10^5xg supernatant fraction obtained from ovarian homogenates 24 - 36 h after injection of PMSG abolished LH-stimulable adenylate cyclase activity at supernatant protein concentration of 2 mg/ml; 42 and 48 h after injection of PMSG the degree of inhibition reduced to 67% and 33%, respectively (Fig. 4). A progressive

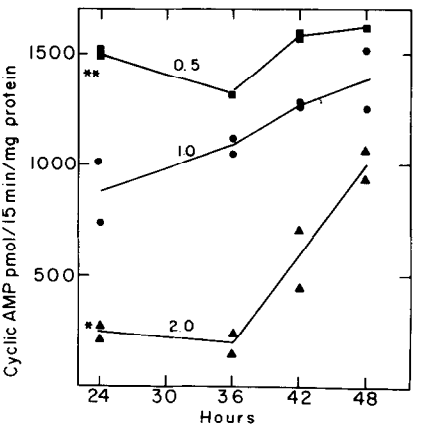

Figure 4. The effect of postmicrosomal supernatant from rat ovarian homogenates on LH-sensitive adenylate cyclase. Ovarian post-microsomal supernatants were prepared at the indicated times after PMSG (15 i.u./rat) injection. These preparations at a final concentration of 0.5, 1.0 and 2.0 mg/ml were included in standard adenylate cyclase assay. Enzyme activity was determined in the presence of 10^{-7} M LH. Each assay system contained 3.0 µg of membrane protein. All other details were as described in the Methods section.

decline of inhibitory activity with time after PMSG injection was also evident using a concentration of 1.0 mg/ml of the soluble fraction; at 0.5 mg/ml no inhibition of the enzyme was detected at any time.

DISCUSSION

It has been suggested that ovarian follicular fluid exerts an inhibitory action on ovum maturation (3) as well as on the process of luteinization in granulosa cell cultures (4). In searching for intra-ovarian factors that may influence ovulation-related events, we found that bovine follicular fluid exerts a direct inhibition on LH-sensitive adenylate cyclase and does so in a dose-dependent manner. Almost complete inhibition was achieved even when using a 8-fold dilution of this fluid (11 mg protein/ml), suggesting that this effect may be of physiological importance. It should however be noted that due to the difficulty of collecting rat follicular fluid, we studied the effect of bovine follicular fluid on rat ovarian plasma membranes. The

results suggest that whatever the nature of the inhibitor, its action is not species-specific.

The molecular weight of the inhibitor has not yet been determined, but is probably higher than 700 daltons, since it was not retained on a Sephadex G-10 column. Furthermore, treatment of follicular fluid with charcoal did not reduce the inhibitory activity thus excluding the possibility that steroids bound to macromolecular components may be involved. It is unlikely that the inhibitor acts directly to neutralize the hormone since inhibition was not abolished at high excess of hormone (Fig. 2 and Fig. 1A).

Sulfated glycosaminoglycans such as heparin, heparan sulfate and dermatan sulfate which are normal constituents of the ovary (5-7), have been shown to inhibit ovarian adenylate cyclase. Heparin, being the most potent of these substances inhibited LH stimulation of adenylate cyclase, but like follicular fluid had only a minute effect on NaF stimulation (8-10). Therefore, the possibility that heparin-like substances contribute to the inhibitory action of follicular fluid should be considered.

The mechanism by which follicular fluid inhibits the LH-sensitive adenylate cyclase is not clear. Since hormone binding is only moderately inhibited at concentrations which block adenylate cyclase activity almost completely, it is not likely that the follicular fluid acts exclusively by interfering with hormone binding. Moderate inhibition of follicle stimulating hormone binding by bovine follicular fluid has recently been reported by Darga and Reichert (13). Enzyme activity stimulable by fluoride was not affected by the follicular fluid (Fig. 1). It is generally believed that fluoride ions stimulate adenylate cyclase directly and not *via* the hormone receptor. This would suggest that the site of inhibition is not the catalytic component of the enzyme. The inhibition by follicular fluid seen in the absence of added LH (Fig. 1A) can therefore be explained as an effect of follicular fluid on a component of adenylate cyclase activity stimulated by

residues of PMSG bound to this membrane preparation. It may be inferred therefore that follicular fluid may interfere with receptor enzyme coupling.

Ledwitz-Rigby *et al.* (4) found reduced activity of a luteinization inhibitor in follicular fluid from larger follicles in the porcine ovary. A reduced concentration of the putative inhibitor would permit increased responsiveness of the follicle to LH towards the time of ovulation. However, further work is required to establish a physiological role for the inhibitor. It is also not clear how the level of the inhibitor is controlled in the gland. Early findings of Zachariae suggest degradation of high molecular weight components of follicular fluid close to ovulation (1). We have recently found that the biosynthesis of sulfated mucopolysaccharides is inhibited by progesterone and LH (5-7).

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