The production of plasminogen activator by ovarian granulosa cells has been previously reported to be temporally correlated with ovulation in the rat and to be under hormonal control of gonadotropins. We have examined the type of plasminogen activator produced by granulosa cells and also investigated other ovarian cell types for synthesis of this enzyme. Using antibodies specific for tissue-type or urokinase-type plasminogen activator, we have found that granulosa cells produce exclusively the tissue-type enzyme. However, in cultures of whole follicles isolated from the ovary, there is primarily synthesis of urokinase-type plasminogen activator. Examination of other isolated ovarian cell types has demonstrated that thecal cells secrete the urokinase-type plasminogen activator and that the production of this enzyme is also regulated by gonadotropins and temporally correlated with ovulation. These results suggest that ovulation requires both types of plasminogen activator and that the neighboring granulosa and thecal cells cooperate to ensure rupture of the follicle wall and unimpeded passage of the ovum into the oviduct.

As early as 1916, Schochet proposed a proteolytic mechanism for the rupture of the ovarian follicle (Schochet, 1916). In recent years, it has become apparent that the proteolytic enzyme detected by Schochet in porcine follicular fluid was probably a component of the plasminogen activator-plasminogen system. There is considerable evidence that links the production of plasminogen activator by ovarian granulosa cells to ovulation (Beers et al., 1975; Strickland and Beers, 1976; Beers and Strickland, 1978; Wang and Leung, 1983), including: 1) the production of the enzyme increases dramatically as ovulation approaches, reaches a maximum around the time of follicle rupture, and declines quickly thereafter; 2) the enzyme is induced only in preovulatory follicles; and 3) the enzyme can be induced in vitro by FSH and LH, gonadotropins known to be indispensable for ovulation.

In recent years, considerable new information has been accumulated concerning the molecular aspects of plasminogen activators. In all mammals examined, there are two types of this enzyme, tPA (Astrup and Permin, 1947; Rijken et al., 1979) and uPA (Williams, 1951; Astrup and Sterndorff, 1952; Sobel et al., 1952), so named for their source of isolation. These were distinguished originally using specific antisera (Kucinski et al., 1968; Aoki and von Kaulla, 1971; Christian et al., 1975; Vetterlein et al., 1980; Wilson et al., 1980; Rijken et al., 1981): they are now known to have different catalytic properties (Thorsen, 1977), and recently the cloning of the cDNA for both human tPA (Pennica et al., 1983) and uPA (Heynke, 1982) has confirmed that the two plasminogen activators are products of different genes.

In light of this new information concerning plasminogen activator types, we have reinvestigated the production of this enzyme in the ovary. This paper documents that both tPA and uPA are produced in the ovary and both enzymes are under gonadotropin control. Granulosa cells secrete only tPA, whereas the major source of uPA in the follicle is the thecal cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rats of the Sprague-Dawley strain were obtained from Charles River. Rat FSH (rFSH-1-6) and LH (rLH-1-6) were obtained from the National Hormone and Pituitary Program of the National Institute of Health. hCG and PMSG were purchased from Organon.

**Methods**

Preparation of Cultures—Female rats, 28 days old, were injected subcutaneously with 5 IU of PMSG in 0.2 ml of 0.9% NaCl. For all of the studies involving in vitro exposure of the cultures to hormones, the animals were sacrificed 48 h after the PMSG injection and cells or follicles collected (PMSG-primed animals). For in vivo stimulation hCG (5 IU) was injected subcutaneously in 0.02 ml of 0.5% NaCl 48 h after the administration of PMSG.

Ovarian cell cultures were prepared by a modification of the method of Crisp and Denys (1975), as described in detail (Beers et al., 1975). Briefly, the contents of individual follicles were expressed into medium, and the cells were collected and cultured at 37°C in a 7% CO₂ atmosphere. Using this procedure, the attached cells are >90% viable, and electron microscopic examination has previously revealed that approximately 90% have an ultrastructural morphology characteristic of granulosa cells (Beers et al., 1975).

Cultures of isolated follicles were prepared from PMSG-primed rats. For studies involving in vitro exposure of the follicles to hormones, Graafian follicles were dissected from the ovaries and cultured at a density of 6 follicles/16 mm well in 0.4 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the indicated concentration of hormone. The follicles were randomly chosen from a pool of 5-6 rats. Harvest fluid was collected 10 h later. For studies involving in vivo stimulation, 5 IU of hCG were injected subcutaneously 48 h after injection of PMSG. Preovulatory follicles were dissected from the ovaries at different times after the adminis-
tation of hCG and placed in culture at a density of 6 follicles/well. Harvest fluid was collected 5 h later and stored frozen at -20°C until assayed for plasminogen activator.

Cultures of thecal tissue were prepared as follows. After dissection of follicles from PMSG-primed rats, the follicles were hemisected using a scalpel blade. Theca tissue was gently scraped to eliminate adhering granulosa cells, transferred to fresh medium, and washed vigorously by pipetting up and down through a large tip of a Pipetman. The tissue was again placed in fresh medium and washed as before; this procedure removes most but not all of the granulosa cells (Erickson and Ryan, 1976). The final thecal tissue was then cut into smaller pieces and the equivalent of 6 follicles cultured in 0.4 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Assays for Plasminogen Activator—Routine assays for plasminogen activator were based on the solubilization of 125I-fibrin in the presence of plasminogen (Unkeless et al., 1973). The preparation of 125I-fibrin-coated multwell plates was exactly as previously described (Strickland and Beers, 1976). Up to approximately 60% of the total substrate solubilized, this assay is strictly linear with respect to enzyme concentration.

For zymography of plasminogen activator, culture fluids were subjected to electrophoresis in the presence of sodium dodecyl sulfate under nonreducing conditions according to the procedure of Laemmli (1970). The plasminogen activators were then visualized by placing the Triton-washed gel on a casein-agar-plasminogen underlay as described previously (Granelli-Piperno and Reich, 1978; Belin et al., 1984). The antisera used were as follows. Mouse uPA was purified from the culture medium of a Lewis lung carcinoma cell line and antiserum raised against the enzyme in rabbits (Marotti et al., 1982). The antiserum against human tPA was prepared in rabbits against enzyme isolated from cultures of HeLa cells. This antiserum exhibits excellent cross-reactivity toward the mouse tPA. Both antisera have previously been shown to be specific in inhibiting either tPA or uPA (Marotti et al., 1982). Samples to be tested were preincubated with the appropriate antiserum for 30 min at room temperature before initiating the assay. Anti-tPA and anti-uPA were kindly provided by Drs. Edmund Waller, The Rockefeller University, and Dominique Belin, University of Geneva, respectively.

RESULTS

The availability of antisera specific for either tPA or uPA allows convenient analysis of the type of plasminogen activator produced by cells or tissues. Therefore, granulosa cells were collected from immature PMSG-primed rats and stimulated in vitro with FSH. Under these conditions, the cells secrete large amounts of plasminogen activator (Beers et al., 1975). As shown in Fig. 1, the enzymatic activity was completely abolished by antibodies against tPA, whereas it was unaffected by anti-uPA. This result was anticipated, since the molecular weight of the granulosa cell PA has been shown to be around 70,000 (Strickland and Beers, 1976), which is the usual size of tPA.

As an alternative to granulosa monolayer culture, it is possible to isolate individual intact follicles and maintain these in vitro. This experimental procedure was appealing, since it might demonstrate how tPA is regulated when granulosa cells are associated with other ovarian cells. When follicles were cultured in the presence of gonadotropin, there was substantial PA found in the medium. However, the vast majority of this enzyme was uPA, as demonstrated by antibody inhibition (Fig. 1). Therefore, each of these cultures produced essentially only one type of plasminogen activator.3 When supernatants from granulosa cell and follicle cultures were mixed and assayed on a casein-agar underlay for plasminogen activator, both tPA and uPA were detected at the expected level (data not shown). This result demonstrates that neither culture fluid contains significant amounts of inhibitors for tPA or uPA and that inhibition of activity cannot explain the absence of activity in either culture.

This result raised several questions: 1) since the granulosa cells produce copious amounts of tPA in monolayer culture, why was none of this enzyme detected in the whole follicle experiment; 2) what was the source in the ovary of the uPA; and 3) was this uPA relevant to ovulation or a constitutive ovarian component.

One possible reason that no tPA was detected was that the granulosa cells were dying inside the follicle or not being reached by the inducing hormone. To address this possibility, follicles were cultured for 8 h in the presence of FSH, and the granulosa cells were then isolated from these follicles and maintained in culture. The amount of tPA made by these cells was compared to that secreted by cells stimulated in monolayer. Fig. 2B shows that the granulosa cells stimulated in the follicle produce tPA after removal from the follicle; although the response to gonadotropin is reduced compared to monolayer cultures at low FSH concentrations (Fig. 2A), at higher concentrations the stimulation was the same. One explanation of the difference at low FSH concentrations may be decreased accessibility of the cells inside the follicle to hormone. These results clearly show that the granulosa cells inside the follicle are alive and capable of responding to FSH. Therefore, there must be another explanation for the lack of tPA observed in the whole follicle cultures (see "Discussion").

The source of the uPA in the ovary was of obvious interest. The granulosa cell layer is surrounded by several layers of thecal cells, some of which are secretory (theca interna) and some of which are fibroblastic (theca externa). Since the thecal cells were the likely source of the uPA, cultures of
FIG. 2. Secretion of tPA by granulosa cells stimulated in monolayer or in whole follicles. Granulosa cells or follicles were treated with the indicated concentration of FSH (ng/ml) for 8 h. A, for granulosa cells, the medium was changed and conditioned medium collected 3 h later. B, for follicles, granulosa cells were isolated from the cultured follicles, a monolayer of these cells was established, and conditioned medium collected 3 h later. Both cultures contained the same number of cells. The conditioned media were assayed for plasminogen activator activity.

FIG. 3. Zymography of plasminogen activator secreted by granulosa or thecal cells. The granulosa (1.5 x 10⁵) or thecal (number of cells from 6 follicles) cells were plated in a 16-mm well and stimulated with 100 ng/ml FSH (for granulosa) or 100 ng/ml LH (for thecal). The conditioned media were collected 12 h later, electrophoresed, and analyzed by a casein-agar underlay as described under "Methods." The gel on the left was placed on an underlay containing antiserum specific for uPA (anti-UK) and that on the right on one containing anti-tPA (anti-TA) and that in the center on one containing no antiserum. Gr, granulosa; Th, thecal.

The thecal cells were prepared and analyzed for plasminogen activator production by sodium dodecyl sulfate-gel electrophoresis followed by visualization of the enzyme by putting the gel on a casein-agar-plasminogen film (Granelli-Piperno and Reich, 1978; Belin et al., 1984). Fig. 3 shows that the thecal cells do secrete plasminogen activator and that this enzyme has an apparent molecular weight of about 45,000, in contrast to the granulosa cell tPA of 72,000. Furthermore, the inclusion of specific antibodies in the casein underlay demonstrates that the thecal cells are secreting uPA in contrast to the granulosa cells (Fig. 3).

FIG. 4. Secretion of uPA by isolated follicles stimulated in vitro. Follicles were treated with the indicated concentration of FSH or LH, and the harvest fluid was collected 10 h later. The conditioned media were assayed for plasminogen activator activity in the presence of enough antiserum for tPA to completely abolish any tPA activity.

The results in this paper raise several questions about the production of plasminogen activator in the ovary. One aspect of consideration is the reason that such small amounts of tPA are found in the medium during whole follicle cultures, even though the data show that the granulosa cells inside the follicle can be reached by hormones and induced to secrete enzyme. One possibility is that secretion of tPA into the limited volume of the follicular fluid results in a high concentration of enzyme, and feedback-suppression of synthesis or secretion occurs. There is some evidence for such a regulatory mechanism with tPA (Kadouri and Bohak, 1983). Another possibility is the production of a molecule by the thecal cells that can suppress enzyme formation by granulosa cells. These questions can be better studied in a reconstituted system using granulosathecal mixtures, which will be experimentally more tractable than the whole follicle.
sulfate peptides (Yanagishita et al., 1979; Yanagishita and Hassall, 1979). The proteoglycans may provide a surface for the activation of tPA yielding intrafollicular plasmin (Beers, 1975), which could then degrade the proteoglycans. A second possibility relates to the fact that after rupture it takes minutes for the ovum to escape from the follicle (Blandau, 1955). Since the thecal layers are highly vascularized, there is bleeding at the time of ovulation, and eventually a clot of blood and follicular fluid forms to seal the opening. Formation of such a clot too early would prevent the escape of the ovum and would be disastrous for fertility. It is possible that tPA may be functioning in a fibrolytic capacity in this instance and preventing premature clot formation. It is interesting to note that the rapid decrease of tPA production by granulosa cells after ovulation (Beers and Strickland, 1978) would allow subsequent coagulation at the opening.

Regardless of the exact roles of these enzymes, it seems likely that the further study of this system will help elucidate how uPA and tPA cooperate in ovulation and may yield general insights into granulosa:thecal interactions in the ovary.

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4 The endocrinology of plasminogen activator regulation in the rat ovary is complex and will be addressed in a subsequent publication (R. Canipari and S. Strickland, manuscript in preparation).
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