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REVIEW

Molecular Mechanism of Follicle Rupture during Ovulation

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INTRODUCTION

The preovulatory surge of gonadotropins induces a series of changes in ovarian follicles [18]. The number of follicles that begin to develop is considerably greater than the number that reach maturity. They may degenerate at any stage of development from primordial follicles, and only one or a few follicles that reach maturity go on to rupture and set free the individual oocyte, enabling it to meet the sperm and become fertilized [8, 19]. Together with the subsequent transformation of the follicles into corpus luteum, this sequence of events is referred to as the ovulatory process. The term "ovulation" should be used to denote the entire sequence of follicular responses to gonadotropins, but instead is generally used for the more restricted phenomenon of follicle wall degradation leading to extrusion of the oocyte. In this review, we confine our discussion to follicle rupture, with particular attention to the role of proteolytic enzymes during ovulation in mammals. From our recent data [17, 42], we propose a modified proteolytic cascade model for follicle rupture.

CHANGES IN THE FOLLICLES

Ovarian follicles are embedded in the stroma of the cortex. Secondary follicles, characterized by the presence of a 2–6 layer membrane "granulosa" surrounding the primary oocyte, develop from primary follicles enveloped by a single layer of cells. While the follicles grow further mainly by increasing in number and in size of the granulosa cells, the stroma immediately surrounding the follicle differentiates into the theca interna and the theca externa [19]. Follicle-stimulating hormone (FSH) stimulates the growth of ovarian follicles, while luteinizing hormone (LH) triggers normal ovulation and luteinization in the mature follicle [13, 18] (Fig. 1)

FOLLICLE RUPTURE DURING OVULATION

Until the early 1960s, it was generally assumed that an increase in intrafollicular pressure was responsible for rupture of the follicles upon ovulation. This assumption was based on the observation that there is a marked increase in the volume of follicular fluid shortly before rupture, largely due to enhanced influx of fluid from the extravascular space. However, Blandau and Rumery [7], Espey and Lipner [15], and Rondell [36] were unable to detect significant changes in the intrafollicular pressure during the rapid accumulation of fluid within the follicle.

A series of experiments conducted by Espey *et al.* [10] examining the fine structure of follicles provided valuable information on the mechanism of ovulation. They observed a loosening of the collagenous connective tissue at the apex of the follicle as ovulation approaches. They also observed that the injection of some proteolytic enzymes into the antrum of a rabbit follicle can cause morphological changes very similar to those seen in normal follicle rupture [11, 14]. These were the first evidence for the idea proposed in 1916 by Schochet [38] that proteolytic activity probably has a significant role in the degradation of the follicle wall during ovulation.

Beers [5] and Beers *et al.* [6] showed that follicle wall and fluid fractions from both rats and cows contained plasminogen activator (PA) activity. They also showed that the

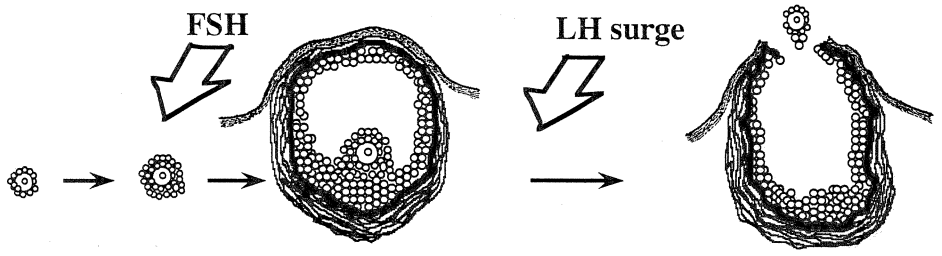

FIG. 1. Follicular maturation and rupture

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activator activity increased in amount toward the time of ovulation and that appearance of the activity was correlated with ovulation. In addition, the presence of plasminogen in the follicular fluid and the reduction of the tensile strength of follicle wall strips by plasmin were demonstrated. Based on these findings, the authors proposed that the PA/plasmin system could be responsible for ovulation. Indirect evidence for involvement of this plasmin-generating system in ovulation was subsequently obtained by pharmacological studies showing that several serine proteinase inhibitors, which strongly inhibit PA and plasmin activities, prevent ovulation in rats *in vivo* [1, 2, 32] and in hamster ovaries *in vitro* [20]. More recently, direct evidence for an essential role of PA and plasmin in ovulation has been provided by an intrabursal injection method using specific antibodies to plasminogen activator and α_2-antiplasmin [44]. Likewise, intrabursal injection of collagenase inhibitors, such as cysteine [33] and talopeptin [20], caused a significant suppression of ovulation, indicating that collagenase(s) plays an important role in follicle rupture as well. According to the current view on follicle rupture, plamsin, the product of PA action on plasminogen, activates latent collagenase(s) and thereby initiates the degradation of the follicle wall [45].

PROTEINASE ACTIVITIES DETECTED IN THE FOLLICULAR FLUID OF PORCINE OVARY

A number of studies on the plasmin-generating system and collagenase(s) have been carried out to elucidate the mechanism of follicle rupture [45]. However, little or no attention has been paid to the activities of other proteolytic enzymes, although a variety of activities have been identified in follicular tissue and fluid [12, 16, 21, 24]. Table 1 shows enzyme activities of follicular fluids obtained from mature follicles of porcine and human ovaries, as assayed with synthetic peptide 4-methylcoumaryl-7-amide (MCA) substrates. The fluids contain enzyme(s) with a cleavage specificity for the Arg-X bond of some substrates. The distribution of the activity toward the substrate Boc-Gln-Arg-Arg-MCA was examined in the porcine ovary. As illustrated in Figure 2, more than 90% of the total activity was recovered from the follicular fluid and its specific activity was much higher than that of the tissue extract [42]. In addition, the activity in the fluid increases as the follicles undergo maturation [42]. The result suggests the biological importance of the enzyme in the events relating to follicular maturation and/or ovulation.

As will be discussed below, the porcine enzyme responsible for the activity towards Boc-Gln-Arg-Arg-MCA, Boc-Gln-Gly-Arg-MCA, and Z-Phe-Arg-MCA was found to be a new serine proteinase capable of activating precursor, single-chain tissue-type plasminogen activator (sc-tPA).

TABLE 1. Enzyme activities of follicular fluids toward MCA substrates

MCA-substrate	pig[a]	human[b]
	nmol/min/ml	
Boc-Gln-Arg-Arg-MCA	29.5	8.52
Boc-Gln-Gly-Arg-MCA	27.3	1.70
Z-Phe-Arg-MCA	29.8	17.7
Boc-Leu-Lys-Arg-MCA	8.8	—
Boc-Val-Pro-Arg-MCA	7.4	—
Boc-Leu-Gly-Arg-MCA	3.5	—
Boc-Gly-Arg-Arg-MCA	1.9	—
Boc-Phe-Ser-Arg-MCA	1.7	—
Boc-Glu-Lys-Lys-MCA	0.28	0.50
Boc-Val-Leu-Lys-MCA	0.20	—
Bz-Arg-MCA	0	—
Suc-Ala-Ala-Pro-Phe-MCA	0.20	—
Suc-Leu-Leu-Val-Tyr-MCA	0.09	0.08
Suc-Gly-Pro-MCA	3.0	—
Gly-Pro-MCA	12.2	—
Arg-MCA	2.6	—
Ala-MCA	3.7	—
Pro-MCA	0.17	—

MCA, 4-methylcoumaryl-7-amide; Boc, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bz, benzoyl; Suc, succinyl.
[a] Fluids from follicles with a diameter >5 mm
[b] Fluids obtained during *in vitro* fertilization procedures. The values represent mean.

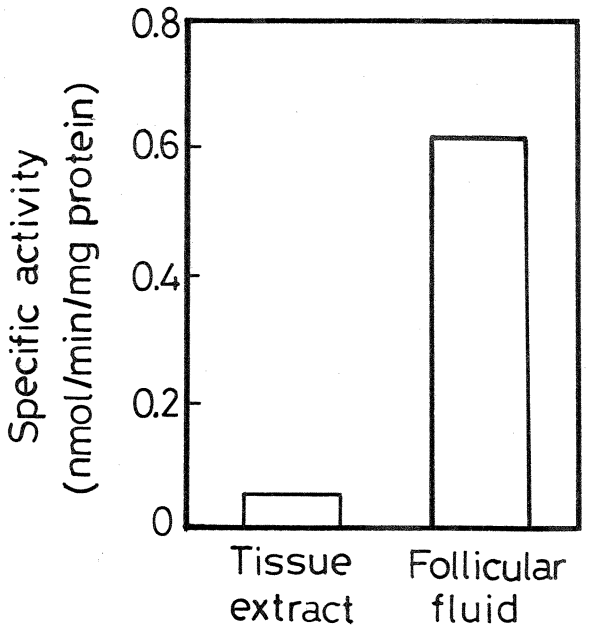

FIG. 2. Enzyme activity in the tissue extract and follicular fluid of porcine ovary. From Takahashi *et al.* [42].

A NOVEL FOLLICULAR FLUID PROTEINASE

Preliminary studies on the follicular fluid enzyme described above suggested that it is apparently distinct from any

of the enzymes known to constitute a proteolytic cascade in follicle wall degradation [42]. To better understand the detailed molecular and enzymatic properties of this enzyme, we undertook its purification and characterization. The enzyme, hereafter referred to as follipsin [17], was purified from the fluid of porcine ovarian follicles by ammonium sulfate fractionation and chromatographies on DE-52, CM-52, and Benzamidine-Sepharose 6B columns. Purified follipsin showed a single polypeptide band (Mr=85,000) in SDS-PAGE under nonreducing conditions, whereas it showed two bands of Mr=45,000 and Mr=32,000 in SDS-PAGE under reducing conditions (Fig. 3). The apparent molecular weight was estimated to be approximately 80,000 by gel filtration on Sephacryl S-200. It is concluded accordingly that the enzyme has a two-chain structure cross-linked by interchain disulfide bond(s). Other molecular and enzymatic properties of follipsin are summarized in Table 2. Although follipsin was shown to be homologous with human plasma kallikrein and factor XIa, its novelty was clearly demonstrated by immunological and chromatographic analyses and substrate specificity studies [17].

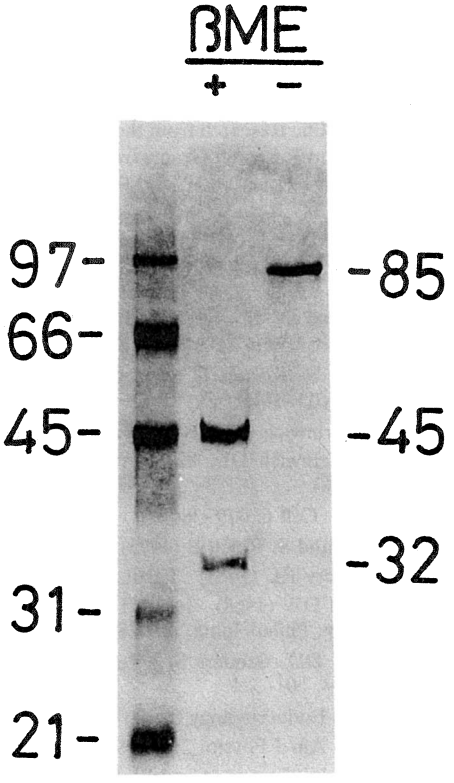

FIG. 3. SDS-PAGE analysis of follipsin. The enzyme was subjected to SDS-PAGE using a 10% gel under reducing (middle lane) or nonreducing (right lane) conditions. Values shown are relative molecular mass (kDa) obtained from the mobilities of standard marker proteins (left lane). β-ME, β-mercaptoethanol. From Hamabata *et al.* [17].

TABLE 2. Molecular and enzymatic properties of follipsin

Molecular weight	80,000 (45,000 and 32,000)
Cleavage specificity	Arg-X bond
Type of proteinase	serine proteinase
Inhibitors	diisopropyl fluorophosphate benzamidine, leupeptin antipain
Other features	accumulation in the antrum during follicular maturation structural homology with human plasma kallikrein and factor XIa

LOCALIZATION OF FOLLIPSIN IN PORCINE OVARY

Immunohistochemical examination of follipsin in ovaries showed that the enzyme is present in the follicular fluid, stroma, and blood vessels (Fig. 4). The positive staining in the follicular cavity is consistent with the biochemical data shown in Figure 2. The localization in the stroma and blood vessels tempts us to speculate that follipsin is originally synthesized in interstitial cells of the stroma of the ovarian cortex and then enters the follicles via the circulatory system. The problems of where the protein is synthesized and of how it reaches the follicular space remain to be answered, however.

ACTION OF FOLLIPSIN ON TISSUE-TYPE PLASMINOGEN ACTIVATOR

In order to dtermine the physiological substrate(s) of follipsin, it is necessary to examine its cleavage specificity in detail using natural peptide substrates. Seven different peptides containing Arg and/or Lys residues were tested with the enzyme (Table 3). Cleavage occurred only on the COOH-terminal side of Arg residues. Interestingly, follipsin rapidly hydrolyzed peptides having a hydrophobic amino acid (Phe, Leu, and Pro) in the P_2 position. In light of such a cleavage specificity of this enzyme, one may consider that follipsin could function as a physiological activator of inactive sc-tPA. The amino acid sequence surrounding the activation site of sc-tPA is -Pro^{272}-Gln-Phe-Arg ‡ Ile-Lys-Gly-Gly-Leu^{280}-, and is perfectly conserved in the proteins from rat [28], mouse [35], and human [30]. This site is apparently a favorable peptide bond for cleavage by follipsin. Follipsin indeed activated tPA efficiently as shown in Figure 5. This activation was accompanied by conversion of inactive sc-tPA (Mr=64,000) into the active two-chain tPA form (2c-tPA, Mr=36,000 and Mr=34,000). It should be noted here that human tPA was used as substrate for porcine follipsin. Experiments using proteins of the same species were not conducted because neither porcine sc-tPA nor human follipsin is available at present.

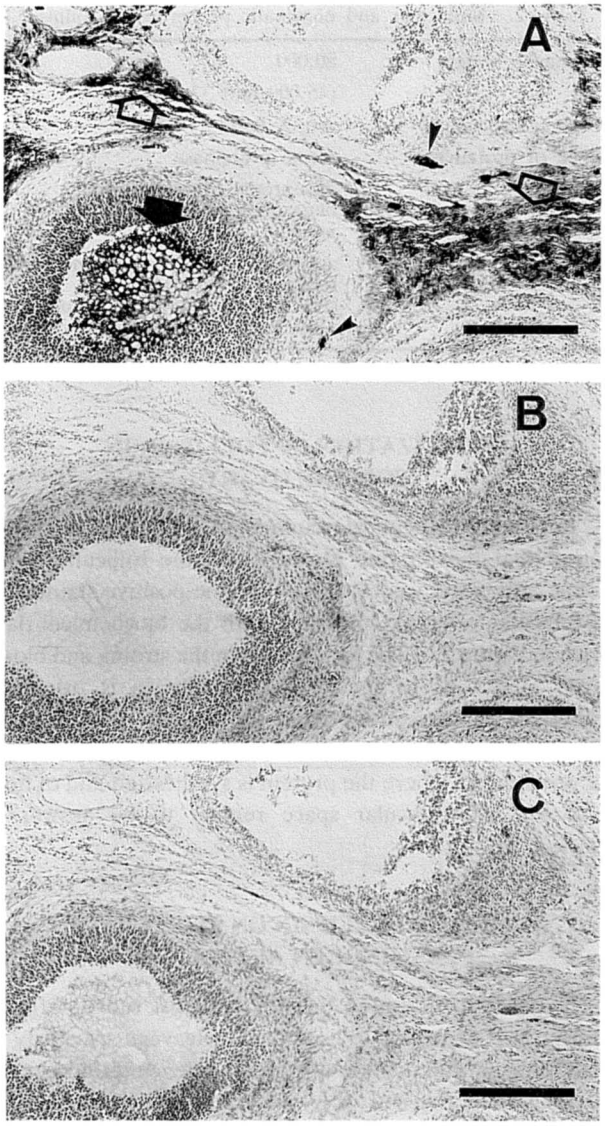

FIG. 4. Immunohistochemical localization of follipsin in porcine ovary. A. Deep color stained with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium system is shown in the follicular fluid (arrow), stroma (open arrow), and blood vessels (arrowhead). B. Normal mouse serum was used instead of anti-follipsin antiserum. C. Immunoneutralized anti-follipsin antiserum was used instead of anti-follipsin antiserum. No positive staining is found in either B or C. Original magnification, × 80. Bar=0.25 mm. From Hamabata *et al.* [17].

A PROTEOLYTIC CASCADE MODEL FOR FOLLICLE RUPTURE

Follicle rupture during ovulation is mediated by a sequential action of proteolytic enzymes constituting a plasmin-generating system and collagenase [45]. The section of Figure 6 enclosed by a solid line has been clearly established by previous studies [5, 6, 45]. More recent studies have demonstrated that tPA is secreted as inactive precursor form (sc-tPA) into the follicular space [25]. Therefore, the activation of tPA must be a prerequisite for initiation of the downstream cascade reaction. Based on our recent finding that follipsin specifically and efficiently activates tPA, we propose its participation in the process of follicle wall degradation, as shown in Figure 6. Single-chain tPA synthesized in and secreted from granulosa cells of mature follicles may be readily activated by follipsin that has accumulated in the follicular fluid. Recently we detected follipsin-like activity in fluids from human ovary obtained during an *in vitro* fertilization procedure and in those of pregnant mare's serum gonadotropin (PMSG)-treated immature rats (our unpublished results). Thus, the model presented here could be a universal proteolytic cascade for follicle rupture in mammals. It should be noted that *in vivo* degradation of the follicle wall is regulated by several specific proteinase inhibitors. α_2-Macroglobulin [24], plasminogen activator inhibitor type-1 (PAI-1) [23, 27, 31, 34], and a tissue inhibitor of metalloproteinase (TIMP) [9, 49] were recenly identified in the follicular fluid of mammalian ovaries.

ROLES OF OTHER FACTORS IN FOLLICLE RUPTURE

The importance of preovulatory progesterone and prostaglandin synthesis in the mechanism of follicle rupture has been suggested. Some studies supporting this idea are summarized in Tables 4 and 5. However, their specific roles remain to be defined.

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TABLE 3. Peptide hydrolysis by follipsin

Peptide	Sequence and cleavage site	Conversion (%)
	↓	
α-MSH	N-Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val	100
	↓	
[Tyr^{38},Phe^{42,46}]-Osteocalcin	Tyr-Glu-Glu-Ala-Phe-Arg-Arg-Phe-Phe-Gly-Pro-Val	100
	↓	
α-Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys	93
	↓	
Neurotensin	<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	85
	↓	
[Asn^{1},Val^{5}]-Angiotensin II	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	14
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0
Serum thymic factor	<Glu-Ala-Lys-Ser-Glu-Gly-Gly-Ser-Asn	0
Mastoparan	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu	0

Peptides (12 nmol) were incubated at 37°C in 40 mM Tris-HCl (pH 8.0) with 5.8 pmol of follipsin in a volume of 100 μl. After two hour incubation, the mixtures were separately subjected to HPLC separation. All peptides collected in HPLC were analysed for amino acid composition. Arrows and the numbers in conversion indicate the sites and extents of cleavage under the condition, respectively.

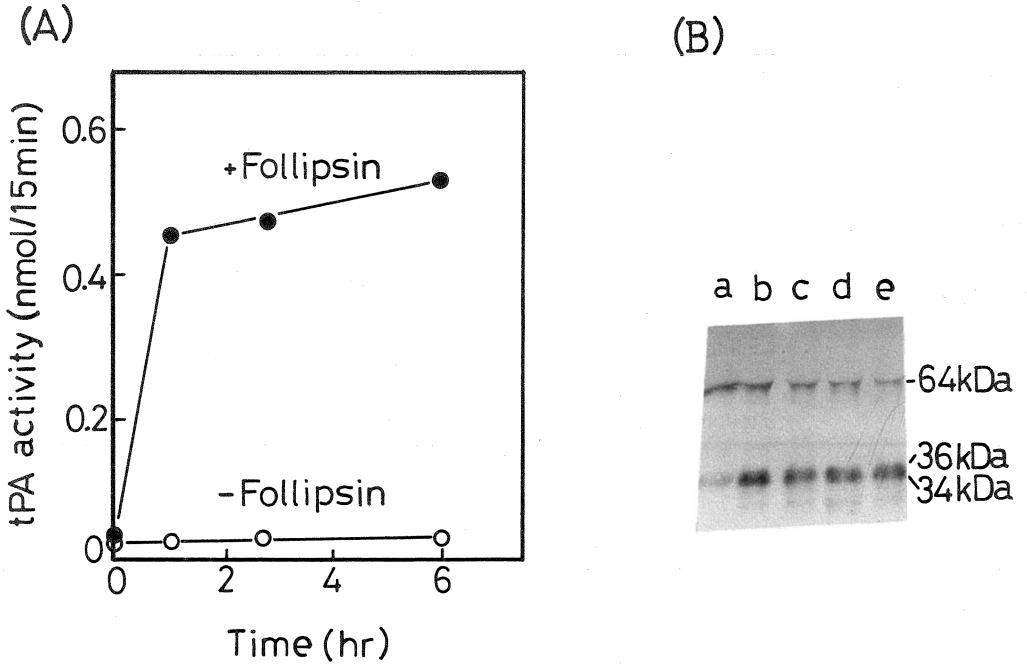

FIG. 5. Activation of tPA zymogen by follipsin. Human single chain tPA (100 pmol) was incubated at 37°C in a volume of 100 μl with follipsin (1.8 pmol) in 50 mM Tris-HCl (pH 8.0). (A) Ten μl of the mixture were drawn at the indicated times and were assayed for tPA activity with Boc-Gln-Gly-Arg-MCA in the presence of proteinase inhibitor aprotinin, which selectively inhibits the activity of follipsin in the samples. (B) Ten μl of the mixture were drawn at 0(a), 1(b), 2(c), 4(d) and 6 hr(e) of incubation for Western blot analysis using anti-human tPA antibody.

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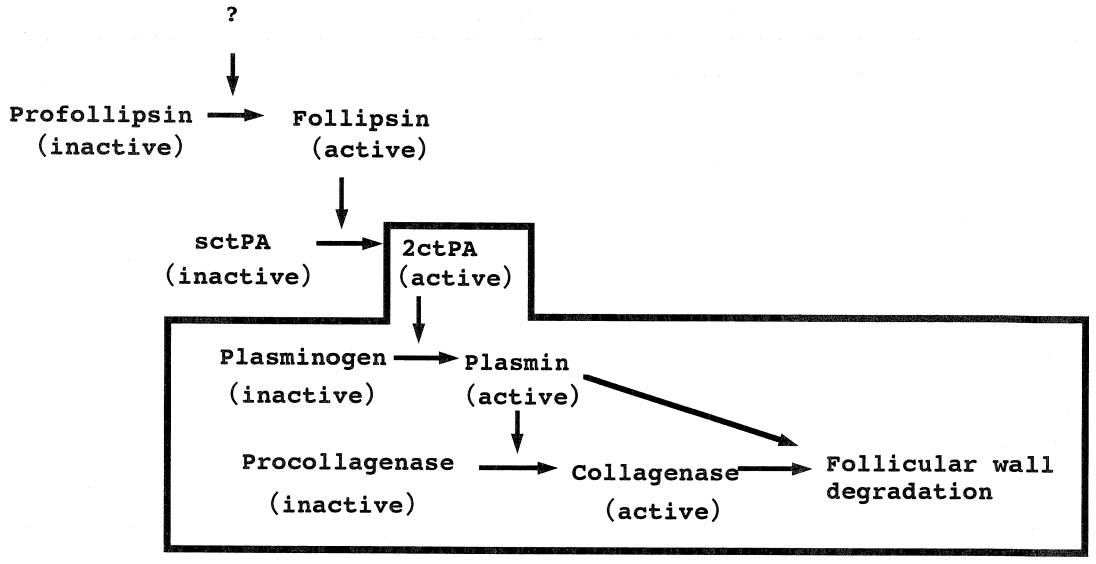

FIG. 6. A model for follicle rupture during ovulation.

TABLE 4. Summary of experiments supporting the implication of progesterone synthesis in follicle rupture

Reagent	Treatment	Effect	Reference
aminoglutethimide and cyanoketone (inhibitors of progesterone synthesis)	administration to ovulation-induced rat	inhibits follicle rupture	37
progesterone	administration to hypophysectomized rat	induces follicle rupture	38
epostane (inhibitor of 3β-OH-steroid dehydrogenase)	administration to rat	inhibits follicle rupture. progesterone overcomes the inhibition	39
isoxazol (inhibitor of progesterone synthesis)	administration to ewe	inhibits follicle rupture	40
RU 38486 (antagonist of progesterone receptor)	administration to rat	inhibits partially follicle rupture	41
progesterone antibody	intrafolliclar injection	inhibits ovulation	42

TABLE 5. Summary of experiments supporting the implication of prostaglandin synthesis in follicle rupture

Reagent	Treatment	Effect	Reference
indomethacin (inhibition of prostaglandin synthesis)	administration to rat	inhibits follicle rupture	43–46
prostaglandin $F_{2\alpha}$ antiserum	intrafollicular injection in rabbit	inhibits follicle rupture	47
prostaglandin E_2, $F_{2\alpha}$	administraion to rat, monkey and ewe	induce ovulation. overcome the inhibitory action of indomethacin	40, 45, 48, 49

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