Stimulation of rhesus monkey (*Macaca mulatta*) proacrosin activation by oviduct fluid

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Summary. Pronase-resistant low molecular weight stimulators for the activation of proacrosin to acrosin were found in rhesus monkey oviduct fluid collected before, during and after ovulation, but the presence of high concentrations of acrosin inhibitors before and after ovulation partly masked the stimulation in unfractionated fluid. This low molecular weight fraction of oviduct fluid had no detectable esterase or amidase activity by itself, and the stimulating factors were sensitive to digestion by hyaluronidase and chondroitin ABC lyase and were presumed to be glycosaminoglycans. Heparin and hyaluronic acid had similar effects. The presence of soluble glycosaminoglycans at the site of fertilization suggests that they may have a role in capacitation and fertilization.

Introduction

Acrosin (EC 3.4.21.10), an acrosomal serine proteinase, is essential for penetration of the zona pellucida by the spermatozoon during mammalian fertilization (see reviews: Fritz *et al.*, 1975; Brown & Hartree, 1976; Morton, 1977; Stambaugh, 1978), and this enzyme exists in the acrosome in a zymogen form called proacrosin. At some time before fertilization proacrosin is activated to acrosin (Meizel, 1972; McLaughlin & Hamner, 1975; Meizel & Mukerji, 1975; Polakoski & Parrish, 1977; Tobias & Schumacher, 1977; Harrison & Brown, 1979), either by autoactivation, or possibly by an activator similar to the bacterial proteinase, thermolysin, reported to be present in testes and spermatozoa (Matsubara, Sasaki, Singer & Jukes, 1966; McRorie, Turner, Bradford & Williams, 1976).

During studies of acrosin inhibitors present in rhesus monkey oviduct fluid (Stambaugh, Seitz & Mastroianni, 1974) it was observed that the low molecular weight fractions of oviduct fluid, collected from the supernatant layer after ultracentrifugation, stimulated rather than inhibited the enzymic activity of some acrosin preparations, apparently due to the activation of some residual proacrosin present in some acrosin preparations. This stimulator of primate proacrosin activation was found to be similar to the hyaluronidase- and chondroitin ABC lyase-sensitive stimulator in uterine fluid that was reported by Wincek, Parrish & Polakoski (1979) and tentatively identified as a glycosaminoglycan. The present study was an investigation of the stimulator in oviduct fluid in rhesus monkeys since the oviduct is the site of fertilization.

Materials and Methods

Chondroitin ABC lyase (EC 4.2.2.4) (from *Proteus vulgaris*), pronase, α -N-benzoyl-L-arginine ethyl ester (BAEE), α -N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), and heparin were purchased from Sigma (St Louis, Missouri). Hyaluronic acid and hyaluronidase were obtained from Worthington, Freehold, New Jersey.

Proacrosin was extracted from ejaculated spermatozoa of rhesus monkeys (*Macaca mulatta*) by a procedure similar to that described by Polakoski & Parrish (1977), but isolated sperm heads rather than whole spermatozoa were used for the extraction. The heads were isolated as previously described using sonication and low-speed sucrose density-gradient centrifugation (Stambaugh & Buckley, 1970), with 50 mM-*p*-aminobenzamidine added to both the sonication medium and sucrose gradients to inhibit autoactivation of proacrosin during isolation. Rhesus monkey spermatozoa were collected by electroejaculation and washed (3 times) with 0.9% (w/v) sodium chloride containing 50 mM-*p*-aminobenzamidine. The washed sperm pellet was dispersed in 4 volumes of 0.9% sodium chloride containing 50 mM-*p*-aminobenzamidine, and the spermatozoa were disrupted by sonication for 1 min at 1 A, 0°C, using a Branson Model LS-75 sonifier with a micro tip. The suspension, in 0.5 ml aliquots, was layered on 10.35 ml linear sucrose gradients containing 65% sucrose at the bottom, and 5% sucrose at the top of the tube in 0.05 M-Tris-HCl buffer, pH 7.2, containing 50 mM-*p*-aminobenzamidine. After centrifugation at 160 g for 10 min, 5°C, head, midpiece, and tail fractions were collected from the bottom of the tube, and the head fractions were pooled and used for proacrosin extraction. Extraction of proacrosin was carried out at 5°C overnight with 10% glycerol containing 50 mM-*p*-aminobenzamidine, pH 3.0. The extracts were centrifuged at 27 000 g for 30 min, 4°C, and the soluble portion was frozen and pooled for further purification immediately before each set of experiments.

Proacrosin was purified from this pooled sperm head extract by an initial dialysis of the extract against 8 M-urea, pH 3.0, 5°C, for 6 h as described by Polakoski & Parrish (1977). This step reversibly denatures the zymogen and removes low molecular weight proteins from the extract. The urea was then removed by an overnight dialysis against 0.25 M-glycine-HCl buffer, pH 3.4, at 5°C. After removal of the denatured proteins by centrifugation (30 000 g), proacrosin was further purified by ultracentrifugation at 216 000 g for 17 h, 5°C, in 0.146 M to 0.584 M-sucrose gradients buffered with 0.25 M-glycine-HCl, pH 3.4. Rhesus monkey proacrosin sedimented in the region of 57 000 molecular weight proteins and appeared to be a single homogeneous fraction. The specific activities for each proacrosin and acrosin preparation are recorded in the tables.

Proacrosin was assayed by determining the amount of acrosin activity produced from the zymogen after autoactivation in 0.15 M-Tris-HCl buffer, pH 8.3. One proacrosin unit was equal to one acrosin unit after autoactivation. Acrosin activity was measured spectrophotometrically by following the hydrolysis of BAEE or BAPNA. The esterolytic activity on BAEE was followed at 254 nm using a Gilford recording spectrophotometer at $25 \pm 0.1^\circ\text{C}$ by the procedure of Bergmeyer (1963). A molar absorbance of $1150 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the amount (μmol) of BAEE hydrolysed. The amidase activity of acrosin was measured spectrophotometrically at 410 nm, 25°C, with BAPNA as the substrate, essentially by the procedure of Erlanger, Kokowsky & Cohen (1961), using an extinction coefficient of 8800 for *p*-nitroaniline. The assay medium contained 9.38×10^{-2} M-BAPNA in 0.05 M-Tris-HCl buffer, pH 8.2, containing 0.01 M- CaCl_2 with a final assay volume of 3.2 ml after the addition of proacrosin or acrosin and the indicated test compounds.

Protein concentrations were determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Rhesus monkey oviduct fluid was collected as before (Stambaugh *et al.*, 1974) by the continuous collection procedure of Mastroianni, Shah & Abdul-Karim (1961) using a refrigerated external collection chamber. Specimens were collected every 24 h before, during, and after ovulation.

The rate of proacrosin activation was measured at 4°C, in 0.025 M-Tris-HCl buffer, pH 7.0, containing 0.1 ml of the low molecular weight fraction of the oviduct fluid or the indicated quantities of glycosaminoglycan activators. The reactions were initiated by the addition of proacrosin at zero time, and 0.05-ml portions were removed for acrosin assays with BAEE or

BAPNA. The results are recorded as specific activities determined from the initial reaction rates of the assay.

Pronase resistance of the stimulatory factor for proacrosin activation was demonstrated by incubating the oviduct fluid with pronase (100 $\mu\text{g}/\text{ml}$) before ultracentrifugation. Pronase sediments into the sucrose gradient during centrifugation at 216 000 g for 17 h and is removed from the low molecular weight fraction in the supernatant layer.

Results

Initial experiments with the low molecular weight fractions from oviduct fluid of preovulatory rhesus monkeys demonstrated a marked stimulation of the activation of proacrosin without loss of the sigmoidal curve characteristic of autoactivation of enzyme zymogens such as trypsinogen and proacrosin (Text-fig. 1). Incubation of 0.1 ml of the supernatant fraction (containing the low molecular weight fractions) after ultracentrifugation resulted in proacrosin activation within 5 to 10 min, a rate 5–6 times the autoactivation time without these fractions. This effect was greatly diminished if the fractions were preincubated with bovine testicular hyaluronidase or chondroitin ABC lyase, but not by pronase (Tables 1 and 2). Unfractionated oviduct fluid collected on the day of ovulation also markedly stimulated proacrosin activation, but unfractionated oviduct fluid collected 3 days before ovulation had a diminished stimulatory effect (Table 3).

Text-fig. 1. Effect of low molecular weight fractions of rhesus monkey oviduct fluid on the conversion of proacrosin to acrosin. The rate of proacrosin activation was measured at 4°C in 0.025 M-Tris-HCl buffer, pH 7.0, containing 0.1 ml oviduct fluid fractions (●), or 0.1 ml water (■). The reactions were initiated by the addition of proacrosin at 0 time, and 0.05 ml portions were removed for assays with BAEE.

The glycosaminoglycans heparin and hyaluronic acid also stimulated proacrosin activation (Tables 1 and 2) and the activation curve was always sigmoidal. Hyaluronidase or chondroitin ABC lyase pretreatment of the oviduct fluid fractions or hyaluronic acid greatly diminished the stimulatory activity of these substances but never completely eliminated the stimulatory effect. Similar results were obtained when BAEE and BAPNA were used to determine acrosin esterolytic and amidase activity, respectively. Control experiments using hyaluronidase or chondroitin ABC lyase in the absence of oviduct fluid or glycosaminoglycans demonstrated that these two enzymes had no effect on proacrosin activation by themselves. Neither the oviduct fluid fractions nor the glycosaminoglycans, heparin and hyaluronic acid, had any significant effect on fully activated acrosin preparations (Table 1).

Table 1. Effects of glycosaminoglycans and low molecular weight fractions (LMF) in oviduct fluid of rhesus monkeys on acrosin and proacrosin

Reactants*	Enzymic activity† (Units/mg protein)
<i>Group 1</i>	
Proacrosin alone	1.9
LMF + proacrosin	98.1
LMF treated with pronase before ultracentrifugation + proacrosin	98.6
LMF pretreated with hyaluronidase (10 µg) + proacrosin	7.6
LMF pretreated with chondroitin ABC lyase (5 µg) + proacrosin	8.4
LMF without proacrosin	0
Activated proacrosin (20 min, 25°C)	102.0
Activated proacrosin + LMF	100.0
<i>Group 2</i>	
Proacrosin alone	1.3
Heparin (10 µg/ml) + proacrosin	91.6
Hyaluronic acid (10 µg/ml) + proacrosin	58.2
Hyaluronic acid (10 µg/ml) pretreated with hyaluronidase (10 µg) + proacrosin	5.2
Hyaluronidase (10 µg) + proacrosin	0.9
Chondroitin ABC lyase (5 µg) + proacrosin	1.0
Activated proacrosin (20 min, 25°C)	95.6
Activated proacrosin + heparin (10 µg/ml)	95.6
Activated proacrosin + hyaluronic acid (10 µg/ml)	95.6

* In 0.15 M-Tris-HCl buffer, pH 8.3.

† Initial reaction rates with BAEE were taken after a 5-min activation period at 4°C. All proacrosin activation experiments with oviduct fluid were carried out with one proacrosin preparation (Group 1). The proacrosin activation experiments in Group 2 were performed with a second proacrosin preparation.

Table 2. Effects of heparin, hyaluronic acid and low molecular weight fractions (LMF) of rhesus monkey oviduct fluid on proacrosin activation using BAPNA for a substrate

Reactants*	Enzymic activity† (Units/mg protein)
<i>Group 1</i>	
Proacrosin alone	2.1×10^{-2}
LMF + proacrosin	510×10^{-2}
LMF pretreated with hyaluronidase (10 µg) + proacrosin	24.3×10^{-2}
LMF pretreated with chondroitin ABC lyase (5 µg) + proacrosin	22.1×10^{-2}
LMF without proacrosin	0
Activated proacrosin (20 min, 25°C).	522×10^{-2}
<i>Group 2</i>	
Proacrosin alone	3.4×10^{-2}
Heparin (10 µg) + proacrosin	483×10^{-2}
Hyaluronic acid (10 µg) + proacrosin	323×10^{-2}
Hyaluronidase (10 µg) + proacrosin	2.6×10^{-2}
Chondroitin ABC lyase (5 µg) + proacrosin	2.4×10^{-2}
Activated proacrosin (20 min, 25°C)	501×10^{-2}

* In 0.15 M-Tris-HCl buffer, pH 8.3.

† Initial reaction rates with BAPNA were taken after a 5-min activation period at 4°C. All proacrosin activation experiments with oviduct fluid were carried out with one proacrosin preparation (Group 1). The proacrosin activation experiments in Group 2 were performed with a second proacrosin preparation.

Table 3. Effects of low molecular weight fractions (LMF) from rhesus monkey oviduct fluid collected before, on the day of and after ovulation on proacrosin activation

Reactants*	Enzymic activity† (Units/mg protein)
Proacrosin alone	1.8×10^{-2}
Preovulatory oviduct fluid + proacrosin	180×10^{-2}
Day of ovulation oviduct fluid + proacrosin	495×10^{-2}
Post-ovulatory fluid + proacrosin	141×10^{-2}
Preovulatory LMF + proacrosin	476×10^{-2}
Postovulatory LMF + proacrosin	482×10^{-2}
Activated proacrosin (20 min, 25°C)	512×10^{-2}

* In 0.15 M-Tris-HCl buffer, pH 8.3.

† Initial reaction rates with BAPNA were taken after a 5-min activation period at 4°C.

Discussion

The experimental results described here demonstrate that the low molecular weight fraction from rhesus monkey oviduct fluid contains one or more components that stimulate the conversion of purified proacrosin to acrosin. The active factor or factors in this fraction of oviduct fluid are sensitive to digestion by both hyaluronidase and chondroitin ABC lyase, but not to pronase digestion. These results are similar to those reported for porcine uterine washings by Wincek *et al.* (1979), who tentatively identified the stimulatory factor(s) as glycosaminoglycans. Unfractionated rhesus monkey oviduct fluid collected on the day of ovulation, when there are low concentrations of acrosin inhibitors (Stambaugh *et al.*, 1974), also markedly stimulated the rate of proacrosin activation, but the stimulatory effect of fluid collected before or after ovulation was not as great. This can be explained by the high concentrations of acrosin inhibitors before and after ovulation that would inhibit autoactivation, since the stimulatory effects of the supernatant fractions after ultracentrifugation are comparable regardless of the day of collection (Table 3).

The demonstration that the common glycosaminoglycans, heparin and hyaluronic acid, also stimulated the activation of primate proacrosin provides additional confirmation that the active components in oviduct fluid are probably glycosaminoglycans, and that the stimulatory effect is not highly specific for the glycosaminoglycan. The lesser stimulation by hyaluronic acid than by heparin suggests that the oviduct fluid stimulators may be sulphated glycosaminoglycans. Chondroitin ABC lyase and hyaluronidase destroyed most of the stimulatory effect of oviduct fluid and hyaluronic acid. The end products of exhaustive digestion of hyaluronic acid by testicular hyaluronidase are tetrasaccharides (90%) and disaccharides (10%). With chondroitin 4- or 6-sulphates the main end products are disulphated tetrasaccharides (Weissman, Meyer, Sampson & Linker, 1954; Mathews & Inouye, 1961). Therefore, it seems possible that the active glycosaminoglycans of oviduct fluid may be sulphated oligosaccharides of unknown structure, but with polymer lengths greater than a tetrasaccharide. Since the sigmoidal shape of the activation curve is retained in the presence of the oviduct fluid-factors, heparin, or hyaluronic acid, the accelerating effect is apparently an acceleration of the autoactivation process. This conclusion is supported by the observations that none of these stimulators had any effect on the enzymic activity of completely activated acrosin preparations, and by the decreased stimulation of proacrosin activation when oviduct fluid containing high concentrations of acrosin inhibitors was used. The stimulation of proacrosin activation is probably, therefore, due to a direct interaction between proacrosin and the glycosaminoglycans. An effect of this type might be mediated by the binding and removal of an inhibitory polypeptide released from proacrosin as this zymogen is activated. Heparin has been reported to accelerate pepsinogen activation by

binding a cationic inhibitory polypeptide released during pepsinogen conversion to pepsin (Harboe, Andersen, Foltmann, Kay & Kassell, 1974), but the release of an inhibitory polypeptide from proacrosin has not yet been demonstrated (Brown & Hartree, 1975).

It is tempting to assign an essential role to glycosaminoglycans in the capacitation process of mammalian fertilization, since glycosaminoglycans are present in uterine and oviduct fluid as well as in the matrix of the cumulus oophorus surrounding the ovum. However, glycosaminoglycans of uterine fluid may never contact the proacrosin of the acrosome since the acrosome reaction takes place in the oviduct and not in the uterus. A physiological role for the glycosaminoglycans now seems to be a definite possibility since they are present in the soluble portion of oviduct fluid at the time of ovulation and in the normal environment created by the oviduct for the acrosome reaction and fertilization.

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