

Inhibition of Bovidae Oocyte Meiosis by Serine Protease Inhibitors (40094)

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It has been known for a number of years that various proteases can induce meiosis in the eggs of marine forms (1, 2). A recent report by Peaucellier (3) indicates that two serine proteases in the digestive tract of the polychaete annelid, *Sabellaria alveolata* initiate meiotic maturation in shed oocytes. The suggestion of Richert and Ryan (4, 5) that serine proteases are involved in the activation of rat ovarian adenylate cyclase by LH prompted the present investigation into a potential role of serine proteases in the re-induction of meiotic maturation in female mammalian systems.

Materials and methods. The method of Jagiello (6) for *in vitro* maturation of Bovidae oocytes was used as the biological assay system. Bovidae oocytes were removed from medium sized follicles and immediately placed into McCoy's media with additives as noted in Tables I-III. They were matured *in vitro* in a constant 5% CO₂/air high humidity atmosphere at pH 7.2 for 24-26 hr. At the end of the incubation the oocytes were prepared for analysis by a modification of the method of Tarkowski (7) and stained with 1% toluidine blue in resin. The attainment of normal first or second meiotic metaphase as scored by cytogenetic examination with a Zeiss 100× bright light objective was considered the end point indicating successful germinal vesicle breakdown and meiotic progression. Cow oocytes, which have a high percentage of maturation *in vitro* in 24-26 hr were used for studying inhibition and conversely, ewe oocytes, which have a moderate percentage, were used for studying meiotic initiation.

The specific site-directed synthetic inhibitors for trypsin, *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK); chymotrypsin, *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma Chemical Company, St. Louis, MO) and elastase, acetylalanyl alanyl

prolyl alanine chloromethyl ketone (prepared by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA), the trypsin substrate analog, *N*-tosyl arginine methyl ester HCl (TAME) (Sigma, St. Louis, MO), the inhibitor of esterases involved in the phosphorylation of serine residues in their active centers, phenyl-methyl sulfonyl fluoride (PMSF) (Schwarz Mann, Orangeburg, NY), and the specific chymotrypsin inhibitor, diphenyl carbamyl chloride (DPCC) (Eastman Kodak Co., Rochester, NY) were studied. Solutions of these agents were freshly prepared in 95% ethanol or DMSO and diluted with media so that the final assay concentrations of alcohol did not exceed 0.025% ethanol or 1% DMSO. Inhibitors in concentrations indicated on Table I were added just prior to the incubation. The natural serine protease inhibitors serum α₁-antitrypsin (National Fractionation Center-American Red Cross, Washington, D.C.), soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), and leupeptin (obtained with the courtesy of Dr. Lowell Greenbaum, Columbia University College of Physicians and Surgeons) were freshly dissolved in media and tested with oocytes at final concentrations noted in Table II. The effects of the three pancreatic serine proteases, trypsin (TRYPTAR) (Armour Pharmaceutical Co., Chicago, IL), chymotrypsin (Worthington Biochemical Corp.) and elastase (Elastin Products, St. Louis, MO), and of a crude human sperm acrosin (prepared by Dr. Wylie Hembree, Columbia University College of Physicians and Surgeons) were dissolved in media and studied in the concentrations noted in Table III.

Results. As seen in Table I, all of the protease inhibitors tested, except the elastase inhibitor, affected cow oocyte maturation *in vitro* ($P < 0.005$) in a dose dependent manner. By cytologic examination the inhibited nuclei

TABLE I. EFFECTS OF SYNTHETIC PROTEASE INHIBITORS ON COW OOCYTE MATURATION.^a

Additive	Final concentration (mM)	Oocytes		
		Total	number matured	%
None	0	64	56	88
TLCK	0.001	49	36	73
	0.01	38	21	55*
	0.1	69	29	42*
	1.0	101	43	43*
	5.0	123	21	17*
None	0	99	79	80
TPCK	0.001	54	33	61
	0.01	80	61	76
	0.1	74	44	59*
	1	80	20	25*
	5	135	17	13*
	None	0	79	64
PMSF	0.001	40	25	63
	0.01	81	51	63
	0.1	71	37	52*
	1.0	40	18	21*
	5.0	87	13	15*
	AcAAPACK	0.00002	92	65
20		50	44	88
None	0	74	61	82
TAME	0.01	41	31	76
	0.1	47	37	79
	1.0	99	45	45*
	5.0	78	31	40*
	DPCC	0.01	60	40
.1		52	28	54*
1.0		34	6	18*
20.0		39	0	0*

^a All cultures were carried out in McCoy's 5A medium (Grand Island Biological Co.)

* $P < 0.005$ inhibition.

TABLE II. EFFECTS OF NATURAL INHIBITORS ON COW OOCYTE MATURATION.

Additive	Inhibitor final concentration per ml	Oocytes		
		Total	Number matured	%
None	0	118	97	82
α_1 -Antitrypsin	5 mg	74	46	62
	10 mg	94	0	0
	5 mg	80	56	70
Soy bean trypsin inhibitor	10 mg	54	34	63
	10 ng	50	34	68
Leupeptin	10 mg	91	70	76

in the presence of each agent had emerged from the germinal vesicle stage and had reached the stage of late condensed chromatin. The approximate concentrations required to inhibit maturation of 50% of the oocytes

relative to the controls were 0.1 mM TLCK and PMSF, 0.3 mM for TPCK, 3.0 mM for TAME and 0.1 mM for the chymotrypsin inactivator DPCC. These approximate potencies were obtained from a logit-log plot of the data in Table I. Solvents alone were without effect. The natural trypsin inhibitor α_1 -antitrypsin was dramatically inhibitory (Table II) at a concentration of 10 mg/ml. Attempts to activate additional meiotic activity above control in ewe oocytes with the pancreatic serine proteases trypsin, chymotrypsin and elastase (Table III) were unsuccessful. However, crude human sperm acrosin was quite inhibitory at concentrations of 1-100 μ g/ml ($P < 0.005$).

Discussion. The inhibition of cow oocyte meiotic maturation by specific inhibitors of serine proteases documented in the present study suggests that a protease or proteases with a similar molecular conformation play a role in the resumption of mammalian meiosis. Since the nuclei attain the early stages of germinal vesicle breakdown and chromatin condensation, it would appear that the initial process of meiotic resumption occurs, but fails to progress when protease activity is inhibited.

Attempts to induce a stimulatory effect on *in vitro* ewe meiosis with pancreatic serine proteases failed, even in concentrations far in excess of those shown to activate the luteal cell adenylyl cyclase system (4, 5). Peaucellier (3) also was unable to mimic the meiosis

TABLE III. EFFECTS OF SERINE PROTEASES ON EWE OOCYTE MATURATION.

Additive	Final concentration (μ g/ml)	Oocytes		
		Total	Number matured	%
None	0	114	86	75
Trypsin	3	78	62	79
	10	78	58	74
	25	32	24	75
	250	91	65	71
	3	41	31	76
α -Chymotrypsin	10	52	38	73
	25	39	28	72
	250	43	27	63
	25	37	24	65
	250	84	57	67
Elastase	25	37	24	65
	250	84	57	67
	1	62	34	55*
Acrosin	10	70	34	49*
	100	52	14	27*

* = $P < 0.005$.

inducing effects of the *Sabellaria* digestive enzymes by equimolar concentrations of commercially available preparations. A different substrate specificity between the pancreatic and ovarian proteases may account for these findings or may be related to an inability of exogenous proteases to gain access to the proper oocyte substrate. The inhibitory effect of the crude human sperm acrosin may be due to acrosin itself or to other contaminating sperm enzymes.

Assays for trypsin-like activity by the technique of Erlanger *et al.* (8) in pools of 200–1000 cow oocytes did not reveal any enzymatic activity. The fact that meiotic maturation is blocked by both trypsin (TLCK, TAME and α_1 -antitrypsin) inhibitors as well as chymotrypsin (TPCK, DPCC) inhibitors suggests either that there is a cascade of proteases or there is a single protease with unique properties. A protease from rat liver has been described that is inhibited by both trypsin and chymotrypsin inhibitors (9). This liver protease also has a highly restricted substrate specificity. The failure to demonstrate protease activity in cow oocytes may therefore be due to an inappropriate substrate.

Bovine and ovine fluids from medium-sized ovarian follicles were found to have trypsin inhibitory activity of 1.70 mg and 1.62 mg trypsin inhibited per ml fluid, a finding consonant with the report of meiotic inhibi-

tion by Bovidae follicular fluid (10) and the data in the present report. The mechanism of action of the specific serine proteases in the re-induction of mammalian oocyte meiosis is being explored in further experiments.

Summary. Specific inhibitors of serine proteases have been shown for the first time to inhibit meiotic progression in mammalian oocytes, suggesting involvement of these enzymes in the process of maturation.

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