Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 4857–4861, November 1977
Biochemistry

Proteolytic enzyme activation of rat ovarian adenylate cyclase

(human chorionic gonadotropin/trypsin/serine proteases/cyclic AMP/luteinized rat ovaries)

NANCY D. RICHERT AND ROBERT J. RYAN

Department of Molecular Medicine, Mayo Medical School and Foundation, Rochester, Minnesota 55901

Communicated by Seymour Lieberman, August 19, 1977

ABSTRACT **Various serine proteases (e.g., trypsin, α-chymotrypsin, Pronase, and subtilisin) stimulate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in a membrane-enriched fraction of the rat ovary. Maximum stimulation is observed at protease concentrations ranging from 3 to 10 μg/ml. Higher protease concentrations inhibit ovarian adenylate cyclase in a dose-dependent manner. Protease stimulation causes a 6- to 8-fold increase in adenylate cyclase activity, which is comparable to the stimulation observed with human chorionic gonadotropin. Combinations of trypsin plus hormone or trypsin plus NaF stimulate ovarian adenylate cyclase activity to a greater extent than does any one of these alone.**

The mechanism of protease stimulation of adenylate cyclase involves limited proteolysis because zymogen precursors fail to activate the cyclase as does trypsin pretreated with trypsin inhibitors. Unlike cholera toxin, the serine protease stimulation is immediate (within the first 5 min) and requires no additional factors (e.g., NAD^+). It is unlikely that protease stimulation of adenylate cyclase results from a proteolytic modification of the hormone receptor on the cell surface, because of the additive effects noted above and because protease stimulation is also observed in ovaries desensitized to hormone that lack this hormone receptor. Results with Lubrol-treated membranes also suggest that proteolytic enzymes do not directly activate the catalytic subunit of the cyclase or unmask new catalytic sites because the protease effect (like hormonal stimulation) is abolished by the detergent, whereas fluoride stimulation is enhanced. Other data suggest that serine protease and chorionic gonadotropin stimulation of adenylate cyclase result from activation of a membrane protease that then regulates adenylate cyclase in the ovary.

Previous studies have demonstrated specific binding of human chorionic gonadotropin (hCG) to the gram-negative bacterium *Pseudomonas maltophilia* (1). We subsequently isolated a protein (molecular weight ~30,000) from the bacterial culture medium that stimulated adenylate cyclase in a membrane-enriched fraction of superovulated rat ovaries (2). Further characterization revealed that the bacterial protein had the properties of a serine protease. In the present study we have examined the effects of other serine proteases, of bacterial or mammalian origin, on rat ovarian adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1].*

MATERIALS AND METHODS

Highly purified hCG-CR121 was a gift from the National Institute of Child Health and Human Development and Robert Canfield, Columbia University. Human thrombin was generously provided by Kenneth Mann, Mayo Clinic. Other enzymes, inhibitors, and reagents were purchased from the following sources: trypsin (type III; 2× crystallized, 11,000 BAEE units/mg), protease (*Streptomyces griseus* type IV, 3.5 units/mg), plasmin (porcine, 0.5 unit/mg), plasminogen, subtilisin BPN′ (type VII, 6.7 units/mg), ovomucoid, phenylmethylsulfonyl fluoride, creatine phosphokinase, creatine phosphate, cyclic AMP (cAMP), bovine serum albumin (Fraction V), pregnant mare's serum gonadotropin, and Lubrol PX from Sigma; α-chymotrypsinogen (5× crystallized), trypsinogen (1× crystallized), and lima bean trypsin inhibitor (LBTI) from Worthington; α-chymotrypsin (bovine 3× crystallized, 1200 NF units/mg) from Schwarz/Mann; Pronase (B grade 45,000 PUK per g) from Calbiochem; 3-isobutyl-1-methylxanthine from Aldrich; and 3a70B liquid scintillation cocktail from Research Products International Corp. *P. maltophilia* protease was purified in this laboratory by Sephadex G-100 and DEAE-cellulose chromatography. Radioactive compounds were obtained from the following sources: $Na^{125}I$ (carrier free) and [α-^{32}P]ATP (10 Ci/mmol) from Amersham; and [^{3}H]cAMP (40 Ci/mmol) from New England Nuclear.

Preparation of 2000 × *g* Ovarian Pellets. Superovulated rat ovaries, at 7–10 days after priming with pregnant mare's serum gonadotropin and hCG, were used for all experiments. Preparation of the membrane-enriched 2000 × *g* pellet fraction has been described (3). The final pellet was resuspended in a 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA and 20% sucrose before storage at −70° for adenylate cyclase assays.

hCG Desensitization of Rat Ovaries. Desensitization was induced by subcutaneous injection of 50 IU of hCG (Ayerst) into superovulated rats 9 days after initial priming with pregnant mare's serum gonadotropin and hCG. Control rats received saline injections. Twenty-four hours later, the ovaries were excised and 2000 × *g* pellet fractions were prepared as described above.

Adenylate Cyclase Assays. Adenylate cyclase activity was determined by the method of Birnbaumer *et al.* (4) as modified by Lee (5) with one further modification. The concentrations of $MgCl_2$ and ATP in the assay were 3 mM and 1 mM, respectively. Hormones and proteolytic enzymes were diluted in 40 mM Tris buffer (pH 7.4) containing 0.1% bovine serum albumin. Incubations were initiated by addition of the membrane preparation and were carried out for 20 min at 30°. Termination of the reaction and isolation of cAMP on Dowex and alumina columns have been described (5). Alumina column eluates were assayed for radioactivity in 10 ml of 3a70B scintillation fluid in a Searle liquid scintillation counter. Cyclase activity was expressed as pmol of cAMP formed in 20 min/mg of protein. Protein determinations were performed by the Lowry procedure (6) with crystalline bovine serum albumin as a standard.

Binding of ^{125}I-Labeled hCG to 2000 × *g* Ovarian Pellets.

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Abbreviations: hCG, human chorionic gonadotropin; cAMP, cyclic AMP; LBTI, lima bean trypsin inhibitor; ^{125}I-hCG, ^{125}I-labeled hCG.

* Part of this work was presented at the 59th Annual Meeting of The Endocrine Society in Chicago, IL (June 8–10, 1977).

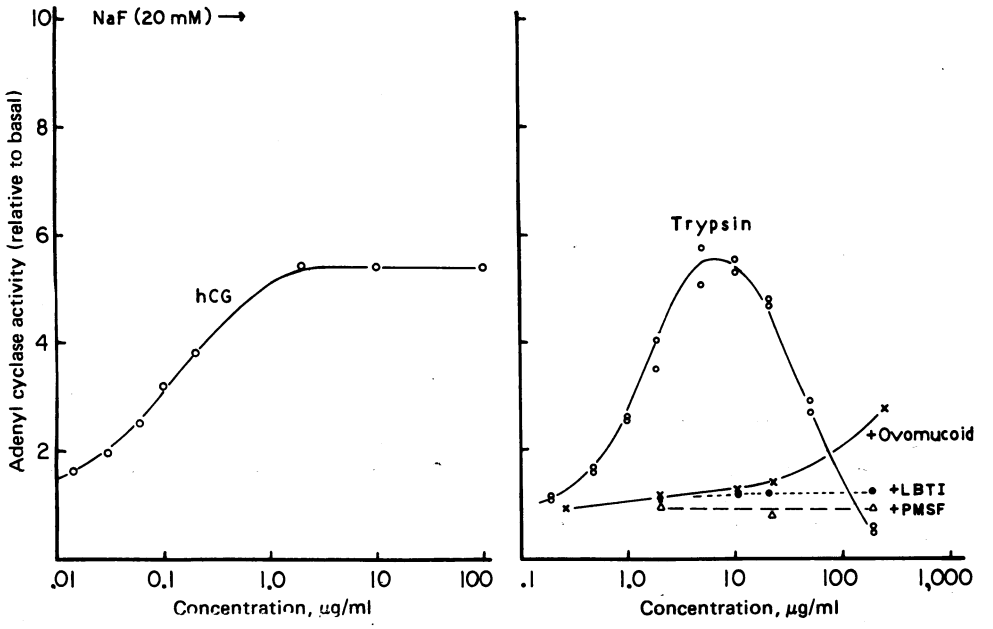

FIG. 1. The membrane-enriched fraction (2000 × *g* pellet) of superovulated rat ovaries was prepared as described in *Materials and Methods*. The reaction mixtures contained ~35 μg of membrane protein, 4 × 10^6 cpm of [α-^{32}P]ATP, 3 mM MgCl, 1 mM cAMP, 1 mM ATP, 1 mM EDTA, 1 mM 3-isobutyl-1-methylxanthine, 20 mM creatine phosphate, 10 μg of creatine phosphokinase, and 25 mM Tris buffer (pH 7.0) in a final volume of 50 μl. The assay concentration of NaF was 20 mM. The final concentrations of hCG (*Left*) and trypsin (*Right*) are indicated. Each data point represents the mean of triplicate assay tubes. The double points for trypsin represent two independent experiments. For trypsin pretreatment with inhibitors, a 2 mg/ml of stock solution of trypsin (in 40 mM Tris/0.1% bovine serum albumin) was incubated in buffer alone or with ovomucoid at 2 mg/ml; (LBTI) at 2 mg/ml, or 1 mM phenylmethylsulfonyl fluoride (PMSF) at 24° for 15 min. The control and treated trypsin stocks were serially diluted in the Tris/0.1% bovine serum albumin and added in 5-μl volumes so that the concentration of trypsin ranged from 10 ng to 10 μg per assay tube. Similar pretreatment of hCG with these inhibitors had no effect on hormonal stimulation of adenylate cyclase.

Preparation of ^{125}I-labeled hCG (^{125}I-hCG) having a specific activity of 40 μCi/μg has been described (3). In the competition assays, increasing concentrations of unlabeled hCG or proteolytic enzymes were incubated with 100 μg of ovarian pellet protein and 2 ng of ^{125}I-hCG in a final volume of 1 ml. The assay buffer consisted of 40 mM of Tris (pH 7.4) and 0.1% bovine serum albumin. Incubations were performed at 37° for 30 min. Assays were terminated by precipitating bound radioactivity with Carbowax (7) or by centrifugation at 2000 × *g* (1). Radioactivity was measured in a Packard autogamma spectrometer.

RESULTS

Effect of hCG and Trypsin on Ovarian Adenylate Cyclase. Increasing concentrations of hCG stimulated ovarian adenylate cyclase in a dose-dependent manner (Fig. 1 *left*). Maximum stimulation (4.42-fold relative to basal; SEM, ±0.16; n = 24) occurred at a hormone concentration of 1 μg/ml. A maximally stimulating dose of NaF (20 mM) resulted in a 9.5-fold (SEM, ±0.33; n = 24) increase in adenylate cyclase activity relative to basal. In 24 assays, basal activity showed a SEM of ±4.9%.

Bovine pancreatic trypsin at a concentration of 5 μg/ml caused approximately a 5-fold increase of adenylate cyclase activity (4.8 ± 0.11, n = 21) (Fig. 1 *right*), comparable to that observed with hCG. Higher trypsin concentrations inhibited adenylate cyclase, and at a trypsin concentration of 200 μg/ml the cyclase activity was less than basal (relative activity <1). This lowering of basal activity has been noted previously and probably represents proteolytic digestion of the catalytic units of the cyclase enzyme (8). Unlike NaF inhibition of adenylate cyclase (8), the trypsin inhibition cannot be reversed by excess Mg^{2+} (30 mM) or by NAD^+ (1 mM). Trypsin pretreated with various inhibitors (ovomucoid, LBTI or phenylmethylsulfonyl fluoride) failed to activate ovarian adenylate cyclase.

Time Course of Ovarian Adenylate Cyclase Activity. Maximally effective stimulatory doses of hCG, trypsin, and the bacterial protease from *P. maltophilia* cultures all showed similar time courses of cyclase stimulation at 30° (Fig. 2). Unlike cholera toxin, which has a 20- to 30-min lag phase, a lag phase of less than 5 min was found with hCG and serine proteases, and adenylate cyclase activity was linear for at least 50 min. In contrast to cholera toxin activation of adenylate cyclase (9), addition of 1 mM NAD^+ did not affect the time course or the degree of stimulation observed with hCG or the serine proteases.

Effect of Other Serine Proteases on Ovarian Adenylate Cyclase. Fig. 3 shows the effect of other bacterial and mammalian serine proteases on ovarian adenylate cyclase activity.

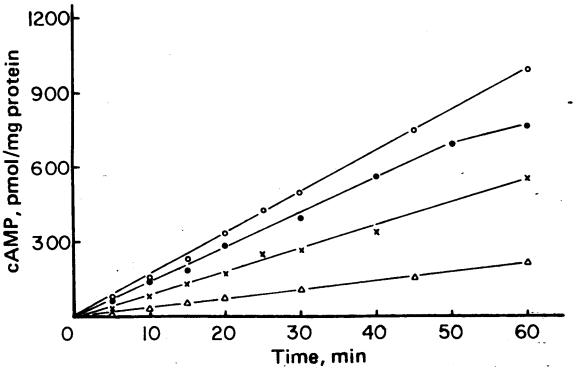

FIG. 2. The reaction mixtures (see Fig. 1 legend) were incubated alone (basal activity) (△) or in the presence of hCG (2 μg/ml) (○), trypsin (5 μg/ml) (●), or the protease from *Pseudomonas maltophilia* cultures (15 μg/ml) (×) at 30° for the times indicated. Each point represents the mean of triplicate assay tubes.

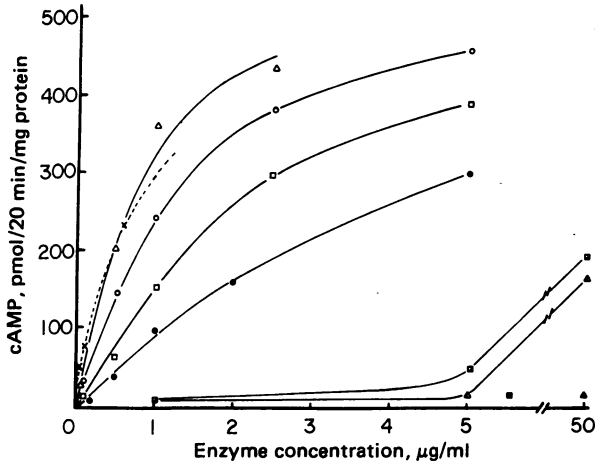

FIG. 3. Increasing concentrations of serine proteases or the zymogen precursors of these proteases were incubated in the adenylate cyclase assays described (Fig. 1 legend) for 20 min at 30°. Symbols are as follows: △, subtilisin; ×, protease; ○, chymotrypsin; □, Pronase; ●, trypsin; ■, plasmin; ▲, chymotrypsinogen; □, thrombin; ▲, plasminogen. Basal cyclase activity (in the absence of added proteases) has been subtracted from each point.

Trypsin, α-chymotrypsin, Pronase, protease (*Streptomyces griseus*), and subtilisin all stimulated adenylate cyclase in a similar manner. Maximal stimulation occurred at protease concentrations ranging from 3 to 10 μg/ml, depending on the enzyme used. Maximal stimulation produced a 6- to 8-fold increase in cyclase activity relative to basal. All of these proteases will inhibit adenylate cyclase at higher concentrations.

Two other serine proteases, human plasmin and human thrombin, were relatively inactive in stimulating ovarian cyclase. A 10-fold higher concentration of plasmin, compared to trypsin, was required for maximal cyclase stimulation. The zymogen precursors α-chymotrypsinogen, plasminogen, and trypsinogen (not shown) were only active to the extent that these commercial preparations are contaminated by active enzymes.

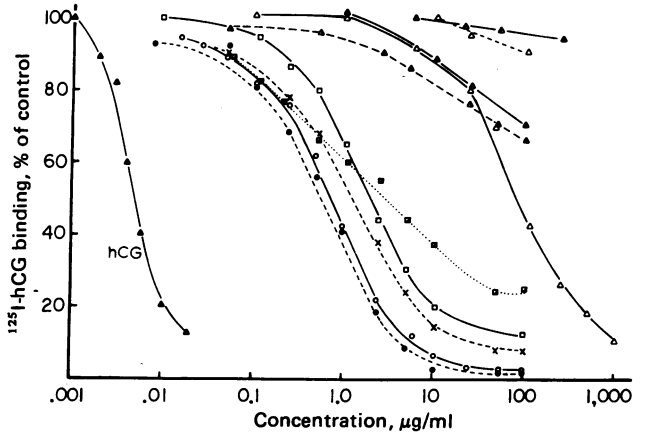

FIG. 4. ^{125}I-hCG (2 ng) was incubated with 100 μg of the 2000 × *g* ovarian pellet and increasing concentrations of unlabeled hCG or serine proteases in 40 mM Tris buffer (pH 7.4) containing 0.1% bovine serum albumin in a final volume of 1 ml. Assays were incubated at 37° for 30 min and terminated with Carbowax (7). Binding of ^{125}I-hCG to the ovarian pellet in the absence of unlabeled hormone or serine proteases was considered as 100%. Absolute binding was 49%. Symbols are as follows: ●, Pronase; ○, protease; ×, chymotrypsin; □, trypsin; ■, subtilisin; △—△, chymotrypsinogen; △, plasmin; ▲, trypsinogen; △- - -△, trypsin and inhibitors; △, plasminogen. Each point represents the mean of duplicate assay tubes.

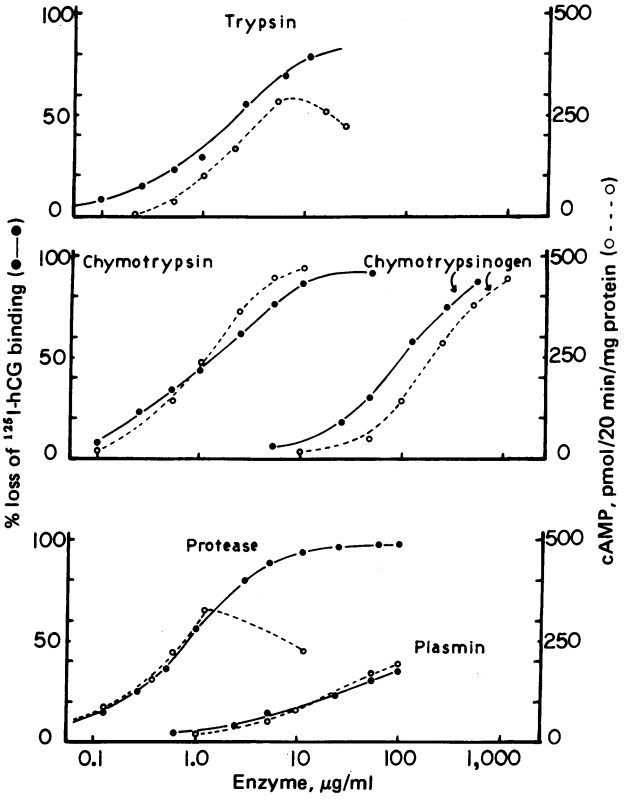

FIG. 5. Inhibition of ^{125}I-hCG binding (from Fig. 4) and of adenylate cyclase activity (from Fig. 3) plotted as a function of serine protease concentration.

Inhibition of ^{125}I-hCG Binding by Serine Proteases. Previous studies by Kono (10) and Kono and Barham (11) suggested that the insulin-like effects of trypsin may result from proteolytic modification of the insulin receptor on the fat cell membrane. To determine whether the serine proteases stimulate adenylate cyclase by a similar specific interaction with the hCG receptor in the ovary, a competition experiment was performed. The results (Fig. 4) show that all the serine proteases that actively stimulate adenylate cyclase inhibit ^{125}I-hCG binding to the 2000 × *g* ovarian pellet in a similar manner. The inhibition of binding is not due to proteolytic digestion of the labeled hormone because the same results were obtained when the ovarian pellet was preincubated with trypsin followed by addition of LBTI (100 μg/ml) to neutralize the trypsin before the addition of labeled hormone. This concentration of LBTI did not affect the binding of ^{125}I-hCG to the pellet.

Plasmin and the zymogen precursors, which did not effectively stimulate ovarian adenylate cyclase, only partially inhibit ^{125}I-hCG binding when added at high concentrations. Similar results were obtained with trypsin pretreated with various inhibitors (LBTI, ovomucoid, or phenylmethylsulfonyl fluoride). α-Chymotrypsinogen inhibited ^{125}I-hCG binding in a manner consistent with the 1% chymotrypsin contamination of this preparation.

For each proteolytic enzyme, the dose-response curve for inhibition of ^{125}I-hCG binding was nearly coincident with the dose-response curve for adenylate cyclase stimulation (Fig. 5).

Protease Stimulation of Adenylate Cyclase in Ovaries Desensitized to hCG. To investigate further the possibility of a specific interaction between the serine proteases and the hCG receptor, rat ovaries desensitized to hCG were used for adenylate cyclase assays. Desensitization was induced by *in vivo*

Table 1. hCG binding activity and stimulation of adenylate cyclase activity in membrane fractions prepared from control rat ovaries and ovaries desensitized to hCG

Rat ovary	Adenylate cyclase activity*					^{125}I-hCG binding, pg/mg
	Basal	hCG	Trypsin	Chymotrypsin	NaF	
Control	130	884	749	1085	1881	140
Desensitized	115	126	644	888	1045	8

2000 × *g* pellet fractions were prepared from control and hCG-desensitized rat ovaries as described in *Materials and Methods*. The membrane preparations were incubated in the adenylate cyclase assay (Fig. 1 legend) alone or in the presence of hCG (2 μg/ml), trypsin (5 μg/ml), α-chymotrypsin (5 μg/ml), or NaF (20 mM). Assays were incubated at 30° for 20 min. Binding assays were done as described in the *text*. All values are means of triplicate determinations.

* As pmol of cAMP/20 min per mg of protein.

injection of 50 IU of hCG. Although the mechanism is not understood, desensitization results in loss of the hCG receptor (12). There was virtually no binding of ^{125}I-hCG to a 2000 × *g* pellet prepared from ovaries desensitized to hCG, and hCG did not stimulate adenylate cyclase (Table 1). In these desensitized ovaries, however, trypsin and α-chymotrypsin still stimulated adenylate cyclase in a manner comparable to that observed in control ovaries. These results suggest that the presence of a functional hCG receptor is not required for proteolytic enzyme stimulation of adenylate cyclase in the rat ovary.

Additive Effect of Trypsin, hCG, and NaF on Adenylate Cyclase Activity. Fig. 6 shows the effect of increasing trypsin concentrations on basal and hCG- and NaF-stimulated adenylate cyclase in the rat ovary. The fact that maximally effective doses of hCG (2 μg/ml) and trypsin had partially additive effects provides further evidence that trypsin does not specifically interact with the hCG receptor to cause cyclase stimulation. The additive effects of trypsin and NaF (20 mM) could be explained if trypsin directly stimulates the catalytic subunit of adenylate cyclase or if trypsin unmasks new catalytic sites. To investigate the latter possibilities, the cyclase assays were performed in the presence of Lubrol.

Effect of Lubrol on Adenylate Cyclase Stimulation. In detergent-solubilized membranes, adenylate cyclase is generally not responsive to hormonal stimulation. Adenylate cyclase can be stimulated, however, by agents, such as NaF, that directly activate the solubilized catalytic subunit of the enzyme (for review, see ref. 13).

The results (Table 2) demonstrate that both hormonal and proteolytic enzyme stimulation of adenylate cyclase were abolished with increasing concentrations of Lubrol. NaF stimulation was slightly increased by the detergent, and basal activity was inhibited at high detergent concentrations. These results suggest that the serine proteases do not directly activate the catalytic subunit of adenylate cyclase in a manner analogous to that of NaF.

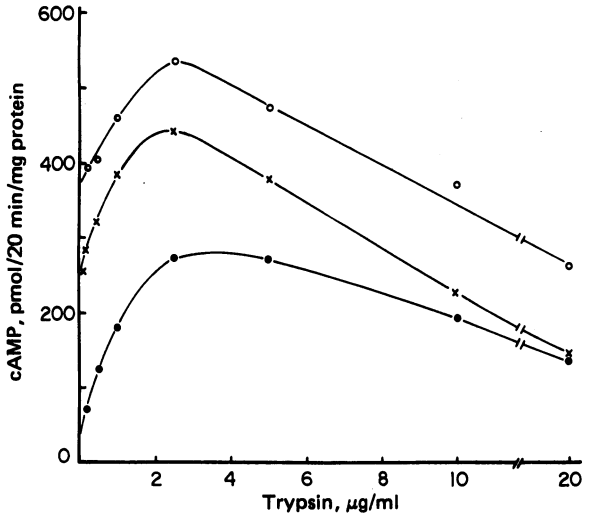

FIG. 6. Increasing concentrations of trypsin were added to the adenylate cyclase incubations alone (●) or together with maximal stimulatory doses of hCG (2 μg/ml) (×) or NaF (20 mM) (○) to determine the effect of trypsin on hormonal and fluoride stimulation of adenylate cyclase. Incubations were for 20 min at 30°. At trypsin concentrations of 200 μg/ml (data not shown), all activities were below basal (i.e., cyclase activity of the membrane preparation in the absence of stimulators). Each data point represents the mean of triplicate assay tubes.

DISCUSSION

The results indicate that various serine proteases stimulate adenylate cyclase activity in a membrane-enriched fraction of superovulated rat ovaries. Maximum protease stimulation causes a 6- to 8-fold increase in adenylate cyclase activity, which is comparable to the stimulation observed with hCG. Results from other laboratories suggest that protease activation of adenylate cyclase is not a phenomenon restricted to the ovary or to broken cell preparations. Hanoune *et al.* (14) recently reported adenylate cyclase stimulation in rat liver membranes by a proteolytic contaminant of crude collagenase. Others have reported that brief trypsinization will increase both basal and fluoride-stimulated adenylate cyclase in a cultured cell line of rat embryo fibroblasts (15). Preliminary experiments in our laboratory suggest that, under defined conditions, trypsin stimulates progesterone synthesis (a cAMP-mediated event) in slices of rat ovary *in vitro*.

Table 2. Effect of increasing Lubrol concentrations on adenylate cyclase activity in rat ovarian membrane preparations

Additions	Adenylate cyclase activity* at various Lubrol conc. (g/dl)					
	0	0.01	0.03	0.05	0.07	0.1
None	53	47	34	35	24	14
NaF (20 mM)	390	378	550	560	512	368
hCG (2 μg/ml)	288	106	35	34	33	20
Trypsin (5 μg/ml)	147	98	43	24	21	13
Chymotrypsin (5 μg/ml)	186	108	77	56	41	32

Increasing concentrations of Lubrol (in 40 mM Tris, pH 7.4) were added to adenylate cyclase incubations containing no stimulators (basal) or to assay mixtures containing NaF, hCG, trypsin, or α-chymotrypsin at the concentrations indicated. The reactions were initiated by addition of the membrane preparation. Assays were incubated at 30° for 20 min. All values are means of triplicate determinations. In a ^{125}I-labeled casein hydrolysis assay, Lubrol has no appreciable effect on trypsin activity (111 ± 5% of control) and increased chymotrpysin activity (206 ± 29% of control).

* As pmol of cAMP/20 min per mg of protein.

Despite the sequence homology among serine proteases, the B chain of cholera toxin, and the β subunits of glycoprotein hormones (16), these agents act via different receptors and/or different mechanisms to activate ovarian adenylate cyclase. Serine protease stimulation requires intrinsic proteolytic activity in the enzyme preparations used. Zymogen precursors (having the same structural homology as the active enzymes) fail to stimulate adenylate cyclase. Furthermore, if the proteolytic activity of trypsin is inhibited by ovomucoid, LBTI, or phenylmethylsulfonyl fluoride, the cyclase-stimulating activity is also lost. In contrast, pretreatment of hCG with protease inhibitors does not affect the cyclase-stimulating properties of the hormone.

Unlike cholera toxin, serine protease stimulation of adenylate cyclase is immediate (within the first 5 min of incubation) and requires no additional factors such as NAD^+. Stimulation of ovarian adenylate cyclase by cholera toxin requires a lag phase of 20–30 min and no stimulation is observed in the absence of 1 mM NAD^+ (data not shown).

It is also unlikely that protease stimulation of adenylate cyclase results from a proteolytic modification of the hCG receptor. Although ^{125}I-hCG binding is inhibited by protease concentrations that activate adenylate cyclase, one cannot determine whether other cell surface receptors are similarly affected. Furthermore, the fact that maximally stimulating doses of trypsin and hCG have partially additive effects suggests that they do not act through a common receptor to stimulate ovarian adenylate cyclase. Finally, trypsin and α-chymotrypsin can still effectively stimulate adenylate cyclase in ovaries desensitized to hCG. This indicates that a functional hCG receptor is not required for protease stimulation.

The results with Lubrol suggest that increased membrane permeability alone cannot account for the stimulation of adenylate cyclase by proteolytic enzymes. Although NaF stimulation is enhanced by the detergent, Lubrol itself does not stimulate basal cyclase activity, regardless of the concentration used. These experiments also indicate that proteolytic enzymes do not directly activate the catalytic subunit of adenylate cyclase or unmask new catalytic sites because the protease effect (like hormonal stimulation) is abolished by the detergent.

The results suggest that the serine proteases must inhibit or activate some other membrane protein which in turn regulates adenylate cyclase activity in the ovary. Because all experiments were performed in the presence of 3-isobutyl-1-methylxanthine and unlabeled cAMP, it is unlikely that protease inhibition of phosphodiesterase could account for the stimulation observed. Furthermore, proteolytic enzyme stimulation of adenylate cyclase in the liver does not result from inhibition of 5′-nucleotidase or ATPase (14).

An alternative mechanism for serine protease stimulation of adenylate cyclase could involve activation of a membrane protease which then stimulates the cyclase. There is increasing evidence that lectin-induced blastogenesis in lymphocytes is mediated by membrane proteases (17–19). Recent studies (20) in our laboratory show that low molecular weight protease inhibitors can selectively block hormonal (hCG) and proteolytic enzyme stimulation of adenylate cyclase without affecting basal or fluoride stimulated activities or ^{125}I-hCG binding. Control studies have ruled out the possibility that the cyclase inhibition results from a direct effect of the inhibitors on the serine proteases or hCG itself. Other studies have shown that these inhibitors can block carbamylcholine stimulation of guanylate cyclase in a mouse neuroblastoma cell line (E. Richelson, N. D. Richert, and R. J. Ryan, unpublished data).

These data suggest that hormonal and proteolytic enzyme stimulation of adenylate cyclase in the ovary, as well as guanylate cyclase stimulation in neuroblastoma cells, may be mediated by membrane proteases. Hormonal activation of a membrane protease could result in adenylate cyclase stimulation and loss of the hormone receptor itself. Thus, protease activation might also explain the hormonally induced down-regulation (desensitization) of the hormone receptors in these tissues.

We are grateful to Kenneth Peters for excellent technical assistance and to Dr. T. A. Bramley for many helpful discussions and suggestions. This work was supported by grants from the National Institutes of Health HD 09140 and HD 01444 and the Mayo Foundation.

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