PROTEASE INHIBITORS BLOCK HORMONAL ACTIVATION OF ADENYLATE CYCLASE

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SUMMARY: To investigate the mechanism of serine protease stimulation of rat ovarian adenylate cyclase, a variety of synthetic protease inhibitors were used. These inhibitors blocked trypsin, chymotrypsin and hCG stimulation of adenylate cyclase in nearly the same manner. The inhibition of hormone stimulated adenylate cyclase could not be explained by a loss of [^{125}I]hCG binding. Cholera toxin and epinephrine stimulation of adenylate cyclase were similarly inhibited, whereas basal and fluoride-stimulated activities were only affected by higher doses of the inhibitors. The results suggest that adenylate cyclase in the ovary may be regulated by membrane protease activity.

We have previously reported that serine proteases (trypsin, chymotrypsin, pronase and subtilisin) stimulate adenylate cyclase (E.C. 4.6.1.1) activity in a membrane-enriched fraction of the rat ovary (1). Protease stimulation causes a 6- to 8-fold increase in adenylate cyclase activity (relative to basal) which is comparable to hormonal stimulation by hCG. The mechanism of protease stimulation is consistent with a limited proteolysis. Detergent solubilization of adenylate cyclase abolishes protease (and hormonal) stimulation, but fluoride stimulation is enhanced. This suggests that proteases do not directly activate the catalytic subunit of the enzyme. Other data suggest that the proteases do not specifically interact with the hCG receptor to activate adenylate cyclase (1).

To further investigate the mechanism of serine protease stimulation, we have used low molecular weight protease inhibitors with different specificities and modes of action. The results demonstrate that a number of these inhibitors not only prevent serine protease stimulation of adenylate cyclase, but also inhibit hCG, cholera toxin, and epinephrine stimulation in the same manner. The results suggest that endogenous proteolytic activity in the cell plasma membrane may regulate adenylate cyclase.

Abbreviations: hCG, human chorionic gonadotropin; cAMP, adenosine 3':5' cyclic monophosphate.

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MATERIALS AND METHODS:

Highly purified hCG (CR 121) was a gift from the NICHD and Dr. Robert Canfield, Columbia Univ. Leupeptin, antipain and chymostatin were provided by Dr. Walter Troll, New York Univ. Medical Center. [α-^{32}P]ATP and [^{3}H]cAMP were purchased from Amersham and New England Nuclear, respectively. All other enzymes, inhibitors and reagents were obtained from Sigma, with the following exceptions: α-chymotrypsin and cholera toxin (Swartz-Mann); pancreatic trypsin inhibitor, and lima bean trypsin inhibitor (Worthington); 3-isobutyl-1-methylxanthine (Aldrich).

Inhibitors. Fresh inhibitor solutions were prepared on the day of the assay. Irreversible inhibitors and L-leucyl-β-napthalamide were dissolved in 95% alcohol, then diluted in 40 mM Tris-0.1% bovine serum albumin (pH 7.4) so that the assay concentration of alcohol did not exceed 3%. This alcohol concentration did not affect basal or stimulated adenylate cyclase activity. All other inhibitor stocks were prepared in 40 mM Tris (pH 7.4).

Adenylate Cyclase Assays. Activity was determined by a modified method of Birnbaumer et al. (2,3). The preparation of the membrane fraction (2,000 x g pellet) from superovulated rat ovaries was described (4). Hormones and proteolytic enzymes were diluted in 40 mM Tris-0.1% bovine serum albumin (pH 7.4) for addition to the assay. Assay concentrations were as follows: hCG (2 μg/ml); cholera toxin (10 μg/ml); epinephrine (0.2 mM); trypsin and chymotrypsin (5 μg/ml); NaF (20 mM). These are maximal stimulating doses.

Assays were initiated by addition of the membrane preparation and were incubated at 30°C. for 20 min. Cholera toxin incubations were performed for 60 min. at 30°C. in the presence of 1 mM NAD+. The termination of the assays, and isolation of cAMP on Dowex and alumina columns have been described (3). Activity was expressed as picomoles of cAMP per mg protein. Protein determinations were performed by the Lowry method (5) using crystalline bovine serum albumin as a standard.

Binding Assays. Preparation of [^{125}I]hCG and binding of the labeled hormone to the 2,000 x g fraction of rat ovarian homogenates have been described (4).

RESULTS AND DISCUSSION:

TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) is an active site titrant and irreversible inhibitor of chymotrypsin that does not affect trypsin (6). In these assays, however, TPCK inhibits both trypsin and chymotrypsin stimulation of adenylate cyclase in the same manner (Fig. 1). Hormonal (hCG) stimulation is similarly inhibited, while basal and fluoride activities are unaffected. If stock solutions (1 mg/ml) of trypsin, chymotrypsin and hCG are pretreated with 5 mM TPCK, then diluted so that the assay concentration of TPCK is 0.025 mM, only chymotrypsin stimulation of adenylate cyclase is inhibited. These results suggest that TPCK does not inactivate trypsin and hCG to prevent stimulation of adenylate cyclase. Other studies showed that TPCK does not inhibit the binding of [^{125}I]hCG to the membrane

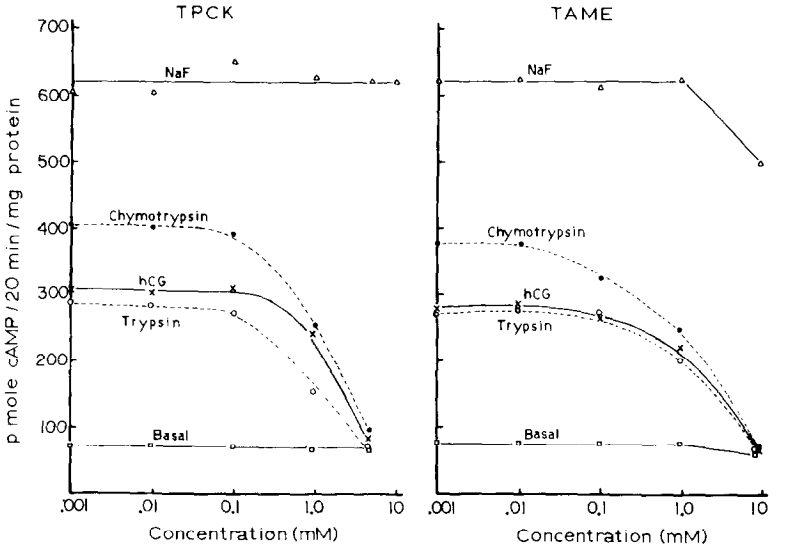

Fig. 1. Effect of increasing concentrations of TPCK and TAME on basal and stimulated adenylate cyclase activity in rat ovarian membranes. In the absence of protease inhibitors, trypsin and chymotrypsin (5 µg/ml) and hCG (2 µg/ml) cause a 4-fold increase in cyclase activity (relative to basal). Fluoride (20 mM) produces a 9.6-fold increase in cyclase activity over basal.

fraction of the rat ovary (Table 1). Thus, TPCK must inhibit some event after hormone binding to block hormonal stimulation of adenylate cyclase.

TAME (p-tosyl-L-arginine methyl ester HCl), a synthetic substrate for trypsin, but not chymotrypsin (7), similarly inhibits adenylate cyclase activation by trypsin, chymotrypsin and hCG (Fig. 1). At higher concentrations, TAME also inhibits basal and fluoride-stimulated activities in absolute terms, but fluoride stimulation relative to basal is unaffected. The fact that TAME inhibition cannot be reversed by higher concentrations of trypsin (up to 2 mg/ml), chymotrypsin, or hCG, suggests that TAME inhibits a membrane enzyme rather than hCG or the added proteases.

Table 1 presents data on a variety of irreversible and competitive inhibitors of serine proteases. At low concentrations, all of the inhibitors affect hCG and protease activation of adenylate cyclase. At higher doses, basal and fluoride stimulation are also inhibited but generally fluoride stimulated activity remains 8- to 12-fold higher than basal. Although maximum

Table 1

Dose Response Effect of Protease Inhibitors on Basal and Stimulated Adenylate Cyclase

Inhibitors	Enzymes[†] Inhibited	Maximum Inhibitor Dose Tested (mM)	ID_{50} for Adenylate Cyclase Stimulation					[^{125}I]hCG Binding[‡] (% of Control)
			Basal	NaF	hCG	Trypsin	Chymo-trypsin	
Irreversible								
MSG	1-5	5	3.0	3.5	0.8	1.5	0.7	56[§]
TLCK[‡]	1,4,5,6	5	0.8	1.0	0.4	0.3	0.3	111
TPCK	2,6	5	*	*	2.5	1.5	1.0	107
Competitive								
BAME	1	10	10.0	(40.0)	2.0	2.0	2.0	94
BAME[‡]	1	10	*	(40.0)	6.0	2.5	9.0	NT
Benzamidine	1	100	100.0	100.0	25.0	5.0	25.0	105
LLβN[‡]	7	2	1.0	2.0	0.2	0.2	0.2	10[§]

Enzyme Code: trypsin(1); chymotrypsin(2); elastase(3); plasmin (4); thrombin(5); sulfhydryl proteases(6); leucine aminopeptidase(7)

TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone HCl; BAME, α-N-benzoyl-L-arginine methyl ester HCl; LLβN, L-leucyl-β-napthalamide

Binding of [^{125}I]hCG, at the maximum inhibitor dose, is expressed as % of control binding in the absence of inhibitors. In control incubations, 49.1% of the total labeled hormone is bound.

No inhibition with PMSF or LLβN was seen at the doses that inhibited stimulation of adenylate cyclase by hCG.

No effect at maximum inhibitor dose.

) Partial inhibition at maximum inhibitor doses. Value in table is extrapolated.

Not tested.

doses of PMSF (phenylmethane sulfonyl fluoride) and L-leucyl-β-napthalamide affect [^{125}I]hCG binding, there is no inhibition of hormone binding at the low inhibitor concentrations which block hormonal stimulation of adenylate cyclase.

The results (Table 2) demonstrate that these protease inhibitors will also reduce cholera toxin and epinephrine stimulation of adenylate cyclase in nearly the same manner as hCG stimulation. Fluoride stimulation, however, is still observed at these inhibitor concentrations. Because many of these

Table 2

Effect of Protease Inhibitors on Cholera Toxin and Epinephrine Stimulation of Adenylate Cyclase

Inhibitors	Dose (mM)	Adenylate Cyclase Activity (cAMP pmoles/min/mg protein)				
		Basal	NaF	hCG	Epinephrine	Cholera Toxin
Control	-	4.7	49.7	18.5	21.2	15.8
PMSF	5	1.7	12.9	1.7	4.1	12.0
TLCK	1	0.7	21.3	0.9	0.8	0.9
TPCK	5	5.2	35.4	5.1	11.8	2.8
TAME	10	2.0	22.7	2.9	6.4	6.4
BAME	10	4.2	35.6	8.4	12.3	10.3
Benzamidine	100	1.8	18.2	2.4	6.9	4.0

Hormone and NaF concentrations were described in Methods. Cholera toxin incubations contained 1 mM NAD+. Because cholera toxin requires a 20 min. lag period for adenylate cyclase stimulation (unpublished observations), these assays were incubated for 60 min. at 30°C. During these longer incubations, the protease inhibitors had a greater effect on basal adenylate cyclase than previously noted in the standard 20 min. assay (Table 1, Fig. 1).

inhibitors are p-toluene sulfonyl compounds, control incubations were performed with toluene and p-toluene sulfonic acid. In contrast to the protease inhibitors, toluene increases protease and hormone-stimulated adenylate cyclase activity (data not shown).

Although inhibitor studies only provide indirect evidence, these data suggest that stimulation of ovarian adenylate cyclase requires endogenous proteolytic activity in the membrane. By inhibiting membrane protease activity, a variety of synthetic protease inhibitors can reduce or eliminate adenylate cyclase stimulation by hormones or exogenous serine proteases. Although membrane proteases have been implicated in lectin-induced blastogenesis (8), cell growth control and contact inhibition (9), the results presented here suggest a more fundamental role for membrane proteases in

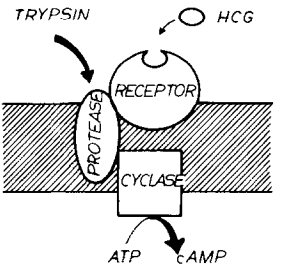

Fig. 2. Proposed model for membrane protease regulation of adenylate cyclase activity.

regulating basal adenylate cyclase activity and its hormonal responsiveness.

Fig. 2 illustrates the manner in which membrane proteases might regulate adenylate cyclase. In the absence of hormones, a low level of membrane protease activity would maintain basal adenylate cyclase activity. The majority of membrane proteases would exist as inactive precursors which could be activated by hormone binding to receptors, or by exogenous proteases. By blocking membrane protease activity, the inhibitors would initially affect hormonal stimulation but would ultimately reduce even basal cyclase activity. Fluoride stimulation could result from a dephosphorylation of the adenylate cyclase catalytic subunit (10), making it more susceptible to proteolytic attack, as shown for phosphorylase kinase (11).

The nature of the membrane protease which regulates adenylate cyclase remains to be determined. The effects of TPCK and TAME suggest that it has both chymotrypsin and trypsin-like properties. A membrane enzyme with similar properties has been described in liver (12). Alternatively, a cascade system involving more than one protease may be responsible for adenylate cyclase stimulation. The unusual properties of the protease itself or its localization in the membrane may explain the failure of natural trypsin inhibitors (ovomucoid, lima bean trypsin inhibitor, pancreatic trypsin inhibitor) and microbial inhibitors (leupeptin, antipain, and chymostatin) to inhibit hormonal stimulation of adenylate cyclase (data not shown).

Further studies are needed to determine whether hCG binding can lead to

protease activation in the ovary. Reichert (13) has shown that *in vivo* injection of pituitary gonadotropins will increase ovarian protease activity. Others have suggested that luteinization is accompanied by a change in ovarian esterase activity (14). If hormone binding leads to activation of a membrane protease, the protease could ultimately be responsible for both adenylate cyclase stimulation and loss of the hormone receptor itself. Thus, hormonal activation of membrane proteases could account for the down-regulation (desensitization) of hormone receptors in the ovary.

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